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Establishing a protocol for the detection of acute myeloid leukaemia markers in liquid biopsies based on extracellular vesicles

Pedro Emanuel Cardoso Nunes

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Pedro Emanuel Cardoso Nunes

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

Pedro Emanuel Cardoso Nunes

Establishing a Protocol for Detection of Acute Myeloid Leukaemia Markers in Liquid Biopsies Based on Extracellular Vesicles

Dissertação de Candidatura ao grau de Mestre em Oncologia com Especialização em Oncologia Molecular submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto.

Orientadora: Professora Doutora Maria Helena Vasconcelos

Professora Auxiliar – Faculdade de Farmácia da Universidade do Porto, Porto

Líder do Grupo "Cancer Drug Resistance" – Instituto de Patologia e Imunologia Molecular da Universidade do Porto / Instituto de Investigação e Inovação em Saúde da Universidade do Porto, Porto

Co-orientador: Professor Doutor Manuel Areias Sobrinho Simões

Médico Assistente Hospitalar – Serviço de Hematologia Clínica do Centro Hospitalar de São João, Porto

Professor Auxiliar Convidado – Faculdade de Medicina da Universidade do Porto, Porto

Investigador afiliado ao Grupo "Cancer Drug Resistance" – Instituto de Patologia e Imunologia Molecular da Universidade do Porto / Instituto de Investigação e Inovação em Saúde da Universidade do Porto, Porto

Co-orientador: Doutor Hugo Ronaldo Caires

Investigador post-doc no Grupo "Cancer Drug Resistance" – Instituto de Patologia e Imunologia Molecular da Universidade do Porto / Instituto de Investigação e Inovação em Saúde da Universidade do Porto, Porto

Experimental activities described in this dissertation were performed at the Cancer Drug Resistance group from the IPATIMUP/i3S institute (Institute of Molecular Pathology and Immunology/Institute for Innovation and Health Research) of the University of Porto (Porto, Portugal). The present work was developed under supervision of Prof. Dra. Helena Vasconcelos (Department of Biological Sciences, Faculty of Pharmacy of the University of Porto) and co-supervision of Prof. Dr. Manuel Areias Sobrinho Simões (Faculty of Medicine of the University of Porto) and of Dr. Hugo Ronaldo Caires (Cancer Drug Resistance)

"Science is based on experiment, on a willingness to challenge old dogma, on an openness to see the universe as it really is. Accordingly, science sometimes requires courage – at the very least the courage to question the conventional wisdom."

Carl Sagan

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List of abbreviatures

AB	Apoptotic Body
AML	Acute Myeloid Leukaemia
ara-C	Cytosine Arabinoside
ATRA	all-trans Retinoic Acid
BM	Bone Marrow
CD	Cluster Differentiation
CHSJ	Centro Hospitalar de São João
CL	Cell Lysate
CLC	Circulant Leukaemic Cell
CR	Complete Remission
D	Diagnosis
DLS	Dynamic Light Scattering
DNA	Deoxyribonucleic Acid
EV	Extracellular Vesicle
FISH	Fluorescence in situ Hybridization
FLT3-ITD	FMS Like Tyrosine Kinase Internal Tandem Duplication
H	Healthy Control
HSC	Haematopoietic Stem Cell
HSP	Heat Shock Protein
IL-3	Interleukin 3
ILV	Intraluminal Vesicle
IPO	Instituto Português de Oncologia do Porto Francisco Gentil

LAIPs	Leukaemia Associated Immunophenotypes
MFC	Multi-parameter Flow Cytometry
miRs	micro Ribonucleic Acid
MRD	Minimal Residual disease
MVB	Multi-Vesicular Body
MW	Molecular Weight
NGS	Next Generation Sequencing
NPM1	Nucleophosmin 1
NTA	Nanoparticle Tracking Analysis
PB	Peripheral blood
PPP	Poor-Platelet Plasma
qRT-PCR	quantitative real time Reverse Transcriptase Polymerase Chain Reaction
Rel	Relapse
RNA	Ribonucleic Acid
RT	Room Temperature
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEC	Size-Exclusion Chromatography
TD-PCR	Tandem Duplication Polymerase Chain Reaction
TEM	Transmission Electron Microscopy
WB	Western Blot
WHO	World Health Organization

Abstract

Acute myeloid leukaemia (AML) is an hematopoietic stem cell disorder defined by a low production of "healthy" hematopoietic cells due to the abnormal accumulation of immature leukaemic cells in the bone marrow (BM). Even though AML only represents 12% of all haematological blood disorders, affecting 156 persons per year in Portugal, this disease has a high mortality rate mainly due to the high frequency of post-treatment relapse.

Thus, assessment of minimal residual disease (MRD) in AML patients is useful to monitor chemotherapy response and to predict which patients are at a higher risk for relapse, therefore allowing the development of personalized treatment regimens. Currently, the MRD monitoring is performed by detection of the remaining leukaemic cells within the AML patients' BM, which severely limits the frequency of disease monitoring. For these reasons, alternative peripheral blood-based methods are highly desirable for an easy, real-time and cost-effective monitoring of MRD in AML.

The work presented in this dissertation aims to demonstrate the feasibility of using extracellular vesicle (EV) - based liquid biopsies for MRD detection. For that, size exclusion chromatography (SEC) technology was implemented to isolate circulating EVs from blood plasma of AML patients. The validation of the SEC isolation method was performed by characterization of the isolated EVs in terms of size, concentration and integrity, by dynamic light scattering (DLS), nanoparticle tracking analysis (NTA) and transmission electron microscopy (TEM), respectively. The EV nature of isolated particles was further confirmed by verifying the expression of HSP70 and CD63 (accepted EV markers) and the absence of cellular debris markers. The purity of EVs eluted in the distinct SEC fractions was also assessed by verifying the presence of albumin.

Importantly, comparison of AML patients' EV profile revealed that complete remission samples have higher EV concentrations than the paired samples at diagnosis. Strikingly, AML patients' circulating EVs carry some of the clinically established AML markers, including CD33, CD34, CD117, CD123, myeloperoxidase, nucleophosmin and fms like tyrosine kinase 3.

In addition, preliminary results indicate that some of these markers, such as CD117, may have potential as MRD biomarkers detected in EVs from blood plasma,

since their levels vary according to the disease status. Moreover, although very low levels of miRs were found in these isolated EVs, preliminary results indicate that the assessment of miR 155 and miR 150 levels in the circulating EVs of AML patients should be further explored for diagnostic purposes. However, further studies are needed to confirm these preliminary results.

Taken together, the work presented in this dissertation established the SEC technology for the isolation of circulating EVs from the plasma of AML patients. Importantly, it was demonstrated that circulating EVs carry some of the clinically established AML markers. Ultimately, this work might contribute to the development of EV-based MRD monitoring, feasible in a clinical setting and holding great promise to impact AML in all stages of the disease.

Keywords: Acute Myeloid Leukaemia, Minimal Residual Disease, Extracellular Vesicles, Biomarkers.

Resumo

A leucemia mieloide aguda (AML) é uma neoplasia com origem nas células estaminais hematopoiéticas, caracterizada pela baixa produção de células hematopoiéticas "saudáveis" devido a uma acumulação anormal de células leucémicas imaturas na medula óssea (BM). Apesar de a AML representar apenas 12% de todas as neoplasias hematológicas em Portugal, afetando cerca de 156 pessoas por ano, esta doença possui um índice de mortalidade bastante elevado devido à elevada frequência de recaídas após o tratamento.

Desta forma, a avaliação de doença residual mínima (MRD) apresenta grande utilidade no acompanhamento clínico de doentes com AML permitindo a monitorização da resposta ao tratamento e a identificação de quais os doentes com maior risco de recaída, possibilitando assim um tratamento mais personalizado. Atualmente, a monitorização da MRD é conseguida através da deteção das células leucémicas residuais na BM de doentes com AML, o que limita seriamente a frequência de monitorização da doença. Por esta razão, existe um grande interesse no desenvolvimento de métodos alternativos para deteção de MRD no sangue periférico para uma monitorização mais intuitiva, eficiente e em tempo real da AML.

O trabalho apresentado nesta dissertação visa demonstrar a utilidade da deteção de MRD mediante análise de vesículas extracelulares (EV) em biopsias líquidas. Para tal, foi implementada a cromatografia de exclusão molecular (SEC) para isolar EVs circulantes do sangue periférico em doentes com AML. A validação do método de isolamento acima referido foi conseguida através da caracterização das EVs isoladas em termos de tamanho, concentração e integridade, mediante a utilização de técnicas como a dispersão dinâmica de luz (DLS), a análise de rastreamento de nanopartículas (NTA) e a microscopia eletrónica de transmissão TEM, respetivamente. A confirmação de que as partículas isoladas correspondem a EVs foi conseguida através da expressão das proteínas HSP70 e CD63 (reconhecidos marcadores de EVs) e ausência de marcadores de detritos celulares. Adicionalmente, verificou-se a pureza das EVs eluídas nas frações da SEC mediante a análise da presença de albumina.

De destacar que a análise do perfil de EVs dos doentes com AML revelou que estes possuem uma maior concentração plasmática de EVs aquando da remissão completa comparativamente ao momento do diagnóstico. De forma

impressionante, as EVs circulantes de pacientes com AML são portadoras de alguns dos marcadores de AML utilizados na prática clínica, incluindo o CD33, CD34, CD117, CD123, mieloperoxidase, nucleofosmina e FLT3.

Para além do referido, resultados preliminares indicam que alguns destes marcadores, com o CD117, poderão vir a ser potenciais biomarcadores de MRD quando detetados em EVs do plasma sanguíneo, tendo em conta que os seus níveis variam consoante o estado da doença. Adicionalmente, e apesar de níveis muito baixos de miRs terem sido detetados nas EVs isoladas, os resultados preliminares indicam que a avaliação dos níveis dos miR150 e miR155 nas EVs circulantes dos doentes de AML devem ser explorados para fins de diagnóstico. Contudo, são necessários mais estudos para confirmar estes resultados preliminares.

No seu conjunto, o trabalho apresentado nesta dissertação estabelece a tecnologia da SEC para o isolamento de EVs circulantes do plasma de doentes com AML. Mais importante, demonstramos que as EVs circulantes transportam alguns dos marcadores de AML utilizados na rotina clínica. Em última análise, este trabalho vem contribuir para o desenvolvimento de uma estratégia de monitorização de MRD com base em EVs, facilmente implementada na prática clínica e com grande potencial para impactar a AML em todas as etapas da doença.

Palavras-chave: Leucemia Mieloide Aguda, Doença Residual Mínima, Vesiculas Extracelulares, Biomarcadores.

Introduction

1. Haematopoiesis

The biological process required for the formation of blood cellular constituents takes place within the bone marrow microenvironment and is called haematopoiesis. The haematopoietic process is a pre-requisite for human embryonic development. During the early stage of development it is vital for production of erythrocytes required for tissue oxygenation, facilitating the rapid growth of the embryo. Later on in development, a more mature and definitive haematopoietic process takes place, to regulate and support the continuous production of all blood cellular constituents of the haematolymphoid system (Jagannathan-Bogdan and Zon, 2013).

The basis of the haematopoietic process resides on the haematopoietic stem cells (HSCs). HSCs are a small cell population localized within stem cell niches in the bone marrow (BM) that are responsible for haematopoiesis due to their self-renewing and multi-lineage differentiation capacities (Weiskopf et al., 2016). HSCs are cells that have the unique ability to differentiate into all blood cell types, which include the erythrocytes, platelets, myeloid lineage cells (monocytes and granulocytes) and lymphoid lineage cells (B cells, T cells and natural killer cells). A haematolymphoid system of a healthy individual generates approximately $5x10^{11}$ mature cells daily, which requires the tight regulation of HSCs proliferation and differentiation by several intra and extracellular factors (Kovtonyuk et al., 2016).

2. Acute myeloid leukaemia

As described, haematopoiesis is a vital process for a healthy haematolymphoid system. Therefore, alterations in this process may result in several blood cell disorders, including life-threatening leukaemias.

Leukaemia in general results from alterations in HSCs or in multipotent haematopoietic progenitors that suffer oncogenic transformation giving rise to leukaemic stem cells. These leukaemic cells have increased self-renewal potential, proliferation, cell survival and/or impaired differentiation (Kennedy and Barabe, 2008). This disease could be further divided and classified according to two important features: leukaemic cell origin and clinical behaviour. The leukaemic cell origin reflects the haematopoietic branch where the leukaemic clone manifests itself. This can be characterized as myeloid, if the leukaemic cells originate in the

myeloid branch, or as lymphoid when the origin of leukaemia resides in the lymphoid branch. On the other hand, the clinical behaviour represents the aggressiveness of the disease, which is intimately related with the leukaemic cell maturation process. Here leukaemia can be broadly divided into acute or chronic, depending on the impact of the oncogenic transformation on the blast cells maturation process. Generally, when a blast cell has an oncogenic event that increases cell proliferation, but has only a mild effect on the maturation process, this gives rise to a chronic leukaemia. Usually, in chronic leukaemia the leukaemic cells continue to partially perform their functions, resulting in milder symptoms, hence their chronic behaviour (Cea et al., 2013; Druker, 2008). However, if the maturation process is severely impaired, the cellular blood constituents will not be able to carry their biological role. Since cellular maturation is affected and as a consequence there is a decrease in the number of functional blood cells, the patients have severe symptoms resulting from anaemia, immune suppression, bleeding and others (Seca et al., 2010). Indeed, the lack of healthy blood cells originates a fast haematopoietic system failure with aggressive symptoms, associated to an acute disease and ultimately (if untreated) to death (Pullarkat and Aldoss, 2015; Kovtonyuk et al., 2016; Kennedy and Barabe, 2008).

Acute myeloid leukaemia (AML) only represents 12% of all haematological malignances, affecting 156 persons per year in Portugal accordingly to the European Cancer Registry (De Angelis et al., 2015). Nevertheless, this disease presents itself as one of the most life-threating types of haematological cancers. These patients (excluding acute promyelocytic leukaemia patients) present a poor 5-year survival of less than 50% (De Angelis et al., 2015). This reality reflects the treatment and follow-up status of European patients with AML. The reason for such critical results lies on the fact that most of these patients will eventually relapse after treatment. This strengthens the need for improvements in AML post-treatment follow-up, through more sensitive and frequent monitoring methods of the disease.

2.1. AML clinical management

During AML development, the patient's peripheral blood composition is altered. Despite the abnormal blood counts, diagnosis requires more sensitive techniques – morphological, immunophenotyping, cytogenetic and molecular genetic analysis are examples of methodologies used to characterize AML. While diagnosis is based

almost exclusively in morphological analysis, prognosis is based on the other techniques (O'Donnell et al., 2017).

2.1.1. Diagnosis, patient stratification & prognosis

The clinician may suspect a diagnosis of acute leukaemia based on the patients' clinical symptoms. This hypothesis is strengthened by abnormal peripheral blood cell counts. Nevertheless, a definitive leukaemia diagnosis can only be made following morphological assessment of the patients' BM (Dohner et al., 2010). This is possible by BM aspiration, that consists in using a needle to aspirate haematopoietic tissue from the marrow cavity of bone.

Regarding the morphological analysis of a BM sample, the World Health Organization (WHO) classification establishes a blast cut-off value of ≥ 20% to diagnose AML. This classification also recognizes a variety of categories that reflect the disease's clinical and biological heterogeneity, including: AML with certain genetic abnormalities, AML with myelodysplasia-related changes, AML related to previous chemotherapy or radiation and AML not otherwise specified (Khaled et al., 2016; Dohner et al., 2017). Indeed, it is clear that AML is highly heterogeneous at the molecular level, as illustrated by the 10 mutations harboured by each AML case on average, with more than 200 recurring balanced chromosomal rearrangements identified in a study of the "Cancer Genome Atlas Consortium" (Cancer Genome Atlas Research et al., 2013). A special AML subtype, where a genetic molecular analysis is mandatory in diagnosis, is acute promyelocytic leukaemia. When the PML-RARα mutation is detected, the administration of all-trans retinoic acid (ATRA) can transform this leukaemia into a higly curable disease. For this special AML subtype, toxic chemotherapies are not necessary. However, a rapid detection of the PML-RARα specific mutation is required (Prada-Arismendy et al., 2016).

Even though morphologic analysis is essential in the clinical diagnosis of AML, the immunophenotype, the cytogenetic and the molecular genetic analysis of the patients' leukaemic blasts are crucial to establish the disease prognosis. Clinical patient stratification and prognosis is required to support clinical decisions on the therapeutic regimen to be adopted. For that, it is critical to gain knowledge on the cellular, genetic and molecular landscape of the AML clones for a given patient. Several technologies are currently available to profile the cellular, genetic and molecular alterations present on these transformed clones. These may include

fluorescence in situ hybridization (FISH), proteomic studies, multi-parameter flow cytometry (MFC), reverse transcriptase-polymerase chain reaction analysis (RT-PCR), gene expression analysis using real-time quantitative RT-PCR (qRT-PCR), PCR for translocations, rearranged or mutant sequences, and DNA sequencing (Hourigan and Karp, 2013; Aasebo et al., 2016).

2.1.1.1. AML cytogenetic and molecular profile stratification

Current clinical guidelines for AML prognosis recognize three risk groups: favourable, intermediate and poor risk. Patients that present at least one of the following genetic abnormalities are included in the favourable risk group: t(8;21), t(15;17), inv(16), t(16;16) or normal cytogenetics accompanied with Nucleophosmin 1 (NPM1) mutation in the absence of FMS-like tyrosine kinase 3 internal-tandem-duplication (FLT3-ITD). Patients who present inv(3), t(3;3), t(6;9), −5, 5q-, −7, 7q-, or complex karyotypes, as well as patients who have normal cytogenetics with a FLT3-ITD mutation, are categorized as poor risk. In the intermediate risk group are those patients with normal cytogenetics and without FLT3-ITD and NPM1 mutations, which correspond to 45% of all AML patients (Prada-Arismendy et al., 2016; O'Donnell et al., 2017).

Recently, analysis of microRNA (miRs) has been proposed as an alternative method to gain knowledge on the leukaemia genetic landscape. These small non-coding RNAs have the ability to regulate gene expression and protein levels by binding to target mRNAs and causing their degradation and/or inhibition of translation (Bell and Taylor, 2017). Thus, these miRs have an important regulatory role over most cellular processes such as cellular signalling, cellular differentiation, proliferation, tumourigenesis and apoptosis. Given their biological role in cell biology and the fact that they are often found deregulated in cancer, it is possible that miRs may become cancer biomarkers (Bellingham et al., 2017; Bell and Taylor, 2017; Shivarov et al., 2014). Importantly, in AML it has been observed that leukaemic blasts have different expression patterns of several miRs when compared to normal counterparts, allowing correlations of risk categories with specific cytogenetic and molecular marker profiles (Lamba et al., 2014; Marcucci et al., 2011).

2.1.1.2. AML lineage stratification

To establish a more accurate clinical prognosis of the disease it is critical to identify the leukaemic blast origin within the haematopoietic differentiation chain. This can be performed with the standard MFC immunophenotype analysis of the blasts present in the BM aspirate sample. This technique is used in clinical routine to identify cells that exhibit leukaemia associated immunophenotypes (LAIPs) (Chen et al., 2015). In principle, the LAIPs are a consequence of altered antigen expression patterns on normal haematopoietic cells, which occurs in up to 90% of AML patients (Tsirigotis et al., 2016; Kayser et al., 2015). These LAIPs are broadly divided into four different categories: cross-lineage expression, overexpression, subexpression and asynchronous expression. The cross-lineage is detected when T-cell, B-cell or NK-cell markers are expressed in myeloid cells, such as: CD2, CD3, CD5, CD19 and CD56; while asynchronous expression is observed when immature (CD34 or CD117) and mature (CD11c, CD14, CD15 or CD65) myeloid antigens are abnormally co-expressed in the same blast cell. The overexpression/subexpression occurs when normally expressed antigens (e.g. CD34, CD123, HLA-DR) are expressed at a higher/lower degree on a leukaemic blast cell when compared to a healthy one (Ommen, 2016; Kern et al., 2010). Usually, in standard MFC analysis, up to 6 or 10 surface protein markers may be used to accurately identify the main haematopoietic lineage subtypes. The incorporation of more antibodies allows increasing the sensitivity and specificity of a MFC determination (Kayser et al., 2015).

2.1.1.3. AML markers

Besides the profiling of surface protein markers by MFC, it is also important to identify other protein and/or nucleic acid AML markers to establish a more accurate clinical prognosis of the disease. Accordingly, in this section the biological role of some of these proteins in “healthy” blast cells will be described. This intends to illustrate how the deregulation of their expression can influence the leukaemia phenotype and ultimately, disease progression, as shown in figure 1.

Some of the proteins that are currently analysed with clinical prognosis purposes are the following:

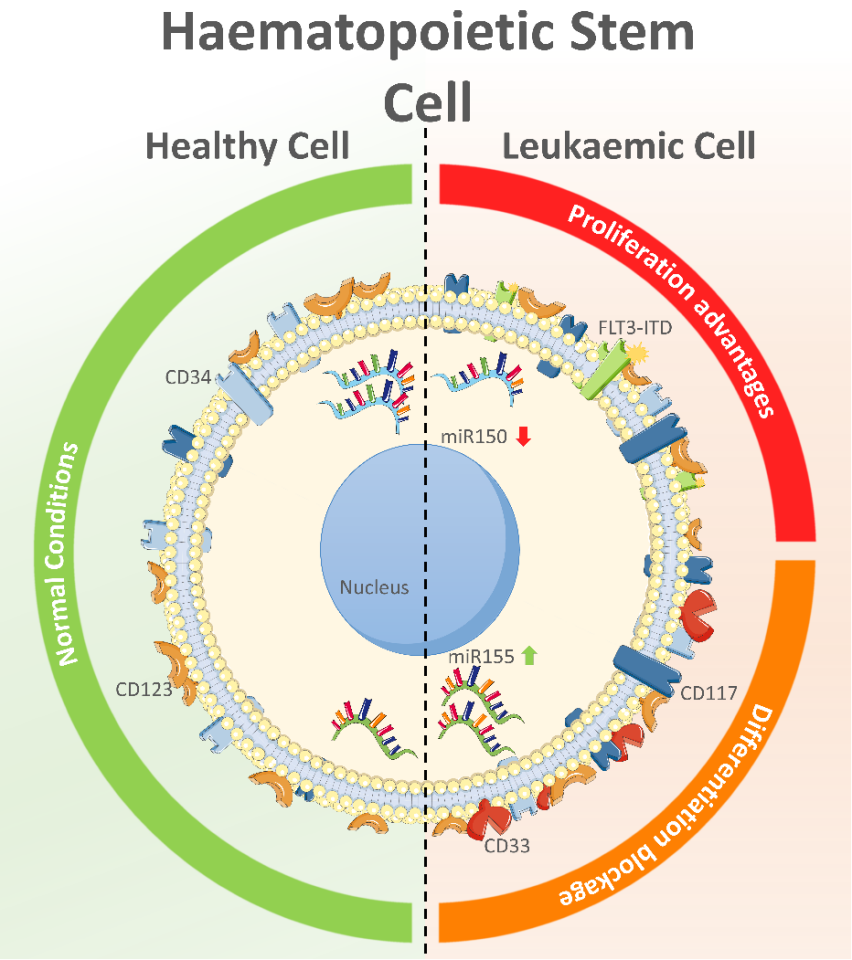

Figure 1 – Differential protein marker expression between healthy HSCs and the counterpart leukaemic blasts. Protein analysis revealed that although CD34, CD117 and CD123 are normally expressed by healthy HSCs, their expression is markedly increased in leukaemic blasts during AML progression. During AML development, other genetic alterations may occur, such as mutation of the transmembranar FLT3 gene originates the mutated protein form, FLT3-ITD. Mutated FLT3-ITD is constitutively activated, generating a continuous signalling for leukaemic blast proliferation. On the other hand, CD33 is a protein related with differentiation that is not expressed in healthy HSCs but is expressed by the majority of leukaemic blast cells. Epigenetic analysis revealed that miR150 and mir155 are consistently dysregulated, being under- or over-expressed in AML, respectively.

NPM1

NPM1 is a phosphoprotein essential for ribosomal protein transport. Once mutated, this protein causes the impairment of the transport of proteins to the nucleus (Copelan et al., 2015). The NMP1 frameshift mutations are one of the most frequent AML abnormalities. Indeed, more than 50 different NPM1 mutations were reported to date, where the three most common variants represent 90% of all mutated cases (Kayser et al., 2015). These mutations have already been studied by qRT-PCR and are classified as being predictive of therapy response (Kronke et al., 2011; Lambert et al., 2014; Hubmann et al., 2014; Karas et al., 2016). Indeed,

levels of NMP1 above 1% after induction therapy are normally associated with high incidence of relapse (Hubmann et al., 2014).

FLT3

FLT3 is another important protein which influences prognosis. FLT3 is a transmembranar tyrosine-kinase receptor that induces cell proliferation following activation by the FLT3 ligand. The ITD mutation causes the permanent activation of FLT3, causing an increase of leukaemic cells proliferation, providing a more aggressive phenotype to the disease (Copelan et al., 2015). In addition to the described methods, it is possible to evaluate these mutations by tandem duplication PCR (TD-PCR), an ultra-sensitive assay designed to detect FLT3-ITD mutations in AML patients (Lin et al., 2015).

CD33

CD33 is an antigen associated with myeloid differentiation. In a healthy donor, CD33 could be found in B-cells, T-cells and in NK cells, but not in HSCs (Walter, 2014). Interestingly, 85-90% of the AML patients' have leukaemic HSCs which express CD33. Therefore, CD33 is used for diagnosis of AML and is a potential therapeutic target (Walter, 2014; Ehninger et al., 2014).

CD34

CD34 is a transmembrane phosphoglycoprotein related to the regulation of differentiation, proliferation and cytoadhesion (even though this last property is somewhat controversial) (Sidney et al., 2014). Clinically, CD34 is associated with HSCs. However, since it is a "stemness" marker it may be found in other non-haematopoietic progenitor cells, such as: stromal, epithelial and endothelial progenitor cells (Sidney et al., 2014). In AML there is an increase immature leukaemic blasts with a phenotype similar to HSCs, leading to an overall overexpression of CD34 in these leukaemic cells. Nevertheless, this marker lacks specificity for AML due to its expression by other non-haematopoietic cells. Therefore, it should not be used as a stand-alone biomarker for AML (Kern et al., 2010).

CD117

The CD117, also named C-KIT receptor, is a transmembranar tyrosine-kinase receptor encoded by the oncogene c-kit. This tyrosine-kinase is activated by

binding to haematopoietic cytokines responsible for the proliferation and differentiation of HSCs. Interestingly, less than 5% of healthy BM stem cells express CD117; however, up to 70% of AML blast cells could express this protein (Liang et al., 2013).

CD123

CD123 is an interleukin-3 (IL-3) receptor that can be found at the cell membrane. IL-3 is a cytokine associated to regulation and production of haematopoietic and immune cells. This IL-3 receptor is expressed in healthy HSCs, with its expression being lost during the differentiation process (Testa et al., 2014). Nevertheless, in AML an overexpression of CD123 protein is generally observed, related with the abnormal increase of immature leukaemic blast cells (Testa et al., 2014; Ehninger et al., 2014).

miR155

In a healthy individual, miR155 is expressed by haematopoietic stem cells and myeloid progenitor cells during inflammatory responses, exerting a regulatory role over the innate immune response. Importantly, miR155 has been suggested as a potential AML biomarker due to its high overexpression in AML patients when compared to healthy controls (Zhi et al., 2013; Marcucci et al., 2013).

miR150

The expression of miR150 during normal haematopoiesis is associated with blast differentiation. However, in leukaemia development it has been described as a tumour suppressor, inhibiting cell proliferation (Li et al., 2017; He et al., 2014). The expression of miR150 was evaluated in AML and was found to be significantly downregulated in those patients, therefore being described as one of the most consistent miR biomarkers for AML (Fayyad-Kazan et al., 2013; He et al., 2014).

2.1.2. Clinical decision algorithm

AML treatment currently consists in one or two cycles of induction chemotherapy until complete remission (CR) is achieved, followed by additional cycles of consolidation chemotherapy. According to the WHO, the clinical state of CR is achieved when ≤5% of myeloblasts are found in AML patient's BM aspirates

(during the BM morphological analysis) and the patient no longer presents AML related symptoms (Dohner et al., 2017).

The basic aim of induction chemotherapy is to induce high levels of cytotoxicity in leukaemic blast cells, causing their death. The specific mechanism of action associated with cell death differs according to the chemotherapeutic drug. In general, these chemotherapy drugs do not target specifically the leukaemic cells. Nevertheless, they display a higher cytotoxicity towards the leukaemic blasts, as these drugs are designed to disrupt different cell pathways which are augmented in the leukaemic cells.

Since this disease has a high proliferative behaviour, the induction chemotherapy usually requires a standard or high-dose of cytosine arabinoside (ara-C) in conjugation with anthracyclines. Ara-C is an antimetabolite, more specifically a cytosine analogue. When incorporated into DNA it causes strand termination, thereby inhibiting cell division and activating a mechanism of cell death by mitotic failure. Anthracyclines are another group of cytotoxic drugs composed by natural products. The anthracyclines mechanism of action is not fully understood but it is known that, like ara-C, they can inhibit cell division by DNA intercalation; in addition, they produce reactive oxygen species that damage the DNA leading to cell death.

The modality of the induction chemotherapy to be selected is decided by the clinician, based on the stratification of the patient's health status. The decision clinical algorithm is exemplified in figure 2. In general, a patient who is less than 60 years-old and with no associated health comorbidities is considered clinically fit to endure the side-effects of a high-dose induction chemotherapy. This high dose chemotherapy treatment consists of a 3h-infusion of high-doses of ara-C twice a day on days 1, 3, 5 and 7. Importantly, patients that are not considered fit for this high dose treatment modality are more suitable candidates to receive a standard dose of induction chemotherapy. This consists of a daily infusion for 10 days with low-dose of ara-C (O'Donnell et al., 2017). However, most of these patients have more than 60 years and/or have serious comorbidities which limit their ability to tolerate the high dose modality. For this clinical decision algorithm,

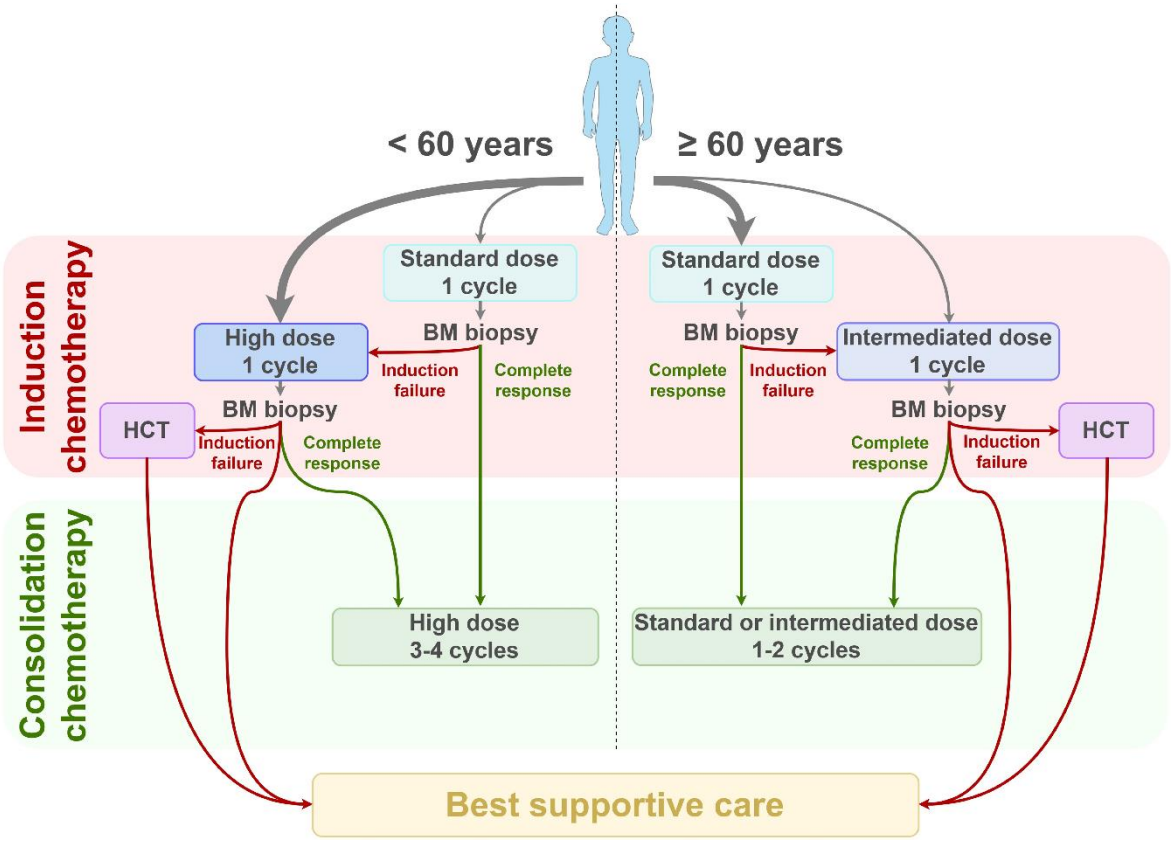

Figure 2 – Clinical decision algorithm for AML treatment. The clinical decision regarding the dose modality of induction chemotherapy prescribed to a given patient is based on the patient's health status. This is usually defined accordingly to the patients age, which is the major factor to consider the patient "fit" to undergo or not a high dose chemotherapy cycle. Although several factors are considered, in order to decide whether the patient is clinically "fit" (or not), the patients' age is the most important factor. Typically, 60 years old is considered to be the cut-off value for this parameter. Other factors such as physical condition and associated comorbidity are also considered. This high dose treatment modality consists of a 3h-infusion of ara-C twice a day on days 1, 3, 5 and 7 plus anthracyclines. Alternatively, for patients who are not considered clinically "fit" to endure a high dose treatment, a cycle of standard-dose is preferred. This standard-dose consists on a daily infusion for 10 days with low doses of ara-C. On the other hand, for patients who are more than 60 years-old, intermediate or a standard dose chemotherapy could be prescribed depending on whether the patient has a favourable physical condition or not. When induction chemotherapy is completed, the patients are submitted to another bone marrow (BM) biopsy to evaluate the disease stage: if a complete response is achieved, the patient continues to consolidation therapy; if not, then a re-induction chemotherapy cycle with higher doses or haematopoietic cell transplantation (HCT) are considered. If the patient fails to achieve complete remission upon re-induction chemotherapy, best supportive care should be offered.

the patient's age is probably the parameter with more impact. Nevertheless, it has to be integrated with a variety of other patient features including the physical condition, which also plays a critical role when deciding the type of treatment modality to be selected. Importantly, due to the conjugation of several factors, exceptions may occur during the course of the clinical decision regarding the dose and number of cycles of the induction chemotherapy.

Even though the induction chemotherapy kills most of the leukaemic blasts, some leukaemic cells can develop mechanisms to evade/survive the drugs administered. In order to minimize the hypothesis of recurrence of the disease, AML patients are submitted to consolidation chemotherapy, which consists in repeating 1 to 4 chemotherapy cycles (O'Donnell et al., 2017). In addition to the consolidation chemotherapy, depending on the patient's health status, an autologous or allogeneic stem cells transplant may be considered in order to eradicate possible residual leukaemic blasts in the BM (Ofran and Rowe, 2015; Maurillo et al., 2015; O'Donnell et al., 2017).

2.1.3. Relapse

After induction chemotherapy, a patient may successfully achieve a clinical state of CR. This is characterized by a significant decrease in myeloblast counts in the BM aspirate sample to less than 5% of the total cell count. Unfortunately, achieving such a clinical state is not a synonym of cure. Notably, even though 50% to 80% of AML patients achieve CR after induction and consolidation chemotherapy, aggressive disease recurrences are likely to occur within the following years. Indeed, accordingly to available AML statistics, younger patients present a 5-year overall survival lower than 50%, while only 20% of the elderly patients survive 2 years after the AML diagnosis (Buccisano et al., 2012; Prada-Arismendy et al., 2016). Considering the high CR rate following therapy and the low 5-year overall survival, one can conclude that a high recurrence rate of the disease significantly contributes to the high mortality rate of AML patients. Indeed, induction chemotherapy may eradicate the majority of the leukaemic clones. Nevertheless, this chemotherapy also exerts a selective pressure on the drug resistant leukaemic clones, which tolerate the toxicity of the treatment. When undetected by current diagnostic techniques and therefore, left untreated, these resistant residual clones will eventually give rise to a recurrence of the disease. This phenomenon happens due to cellular alterations that confer a selective advantage to particular leukaemic "sub-clones". Most frequently, such advantage during treatment is conferred by cellular alterations that decrease drug uptake or decrease the intracellular concentration of drug to sub-lethal amounts. One example is the occurrence of relapse, refractory to conventional chemotherapy drugs, due to the overexpression of drug efflux pumps which prevent the cytotoxic action of some drugs (Holohan et al., 2013; Sousa et al., 2015). Unfortunately, AML patient management in clinical

practice is limited by the lack of accurate methods to detect and monitor in real-time these residual leukaemic cells that survive induction chemotherapy.

Indeed, regarding AML disease monitoring, the majority of protocols and international guidelines relies on BM aspirates as a tool to evaluate the response immediately after induction therapy (Pullarkat and Aldoss, 2015). However, only a small sample of BM is actually examined, which may not reflect the potential involvement of the entire BM compartment. Other limitation of this approach is related to the morphological analysis, since pathologists may not distinguish normal from leukaemic myeloblasts with high accuracy. The criteria to clinically establish morphological CR, which is that it is necessary to have less than 5% of myeloblasts in the BM aspirate samples, is rather inefficient to predict disease recurrence. The inability to distinguish the healthy myeloblasts from leukaemic ones raises the possibility that most of the 5% remaining myeloblasts could be mostly leukaemic clones. In this sense, a more sensitive and specific methodology to evaluate the complete remission would greatly improve AML management (Hong, Muller, Whiteside, et al., 2014).

3. Minimal residual disease: concept and importance in the clinical practice

Minimal residual disease (MRD) is a term used to refer to the disease that cannot be detected by morphological analysis, but can be detected by more sensitive methods. However, this term which was established in 1980, has obtained no consensus nowadays. Indeed, minimal disease cannot be perceived as a clinical steady state, due to the fast disease progression. Importantly, the use of a MRD monitoring in AML could contribute to predict patient's relapse. Nevertheless, caution needs to be taken. One of the main issues to consider is the fact that MRD is a "measurable disease" and therefore the results obtained for a given patient may widely vary depending on the sensitivity of the methodology used for MRD monitoring (Hourigan et al., 2017).

Importantly, several studies have demonstrated that by using high-sensitivity measurements of MRD in AML patients, it is possible to predict outcomes, guide therapeutic interventions or even anticipate relapse in patients who are at a higher risk (Boyiadzis and Whiteside, 2016). Therefore, efforts have been made to develop improved sensitive molecular techniques to allow the accurate detection of MRD. The major difficulty, which is due to the high disease heterogeneity, lies in finding

a reliable MRD biomarker with relapse predictive capacity. Ideally, a MRD marker needs to be sensitive to low disease levels and the used methodology for MRD monitoring must be suitable for frequent analysis. Another problem related to MRD monitoring of AML is the lack of standardization between hospitals, which makes it difficult to compare the sensitivity and specificity of the used protocols. Thus, it is necessary to optimize and standardize the current MRD detection methodologies, aiming to improve current clinical protocols.

3.1. Conventional MRD monitoring

Many haematologists use the MRD endpoint as a marker of disease risk. Indeed, accumulated evidence suggests that AML relapse can be avoided with an early detection of "low-volume" of disease, allowing the anticipated prescription of personalized treatments to prevent recurrence. Therefore, the development of novel MRD detection methodologies, with improved sensitivity down to the molecular level, became critical for improved MRD detection of "low-volume" leukaemic populations (Tsirigotis et al., 2016).

The immunophenotype and molecular genetic analysis, by MFC and PCR respectively, are two of the methodologies in current clinical use for the determination of MRD. However, it is important to point out that these techniques measure two different parameters. MFC detects the malignant cells by identifying specific proteins at the cells surface which are associated with the disease. Indeed, the ability to detect an increase in the expression of immature cell markers in comparison to healthy donors allows following the disease progression and predicting when these leukaemic cells will cause a disease recurrence at the morphological level. On the other hand, PCR-based MRD measurements are performed by evaluating specific genes or mRNA sequences that are related to the transformation of leukaemic cell activity. The FLT3-ITD, as mentioned above, is an example of one mutation with high predictive value for AML. Accordingly to Ommen, the sensitivity and specificity of both methods (MFC and PCR) seems to be similar for MRD determination. Nevertheless, it is still unclear which technique provides a better discrimination between risk groups (Ommen, 2016).

One disadvantage of these techniques to monitor MRD in the clinical practice resides in the fact that both require the use of BM samples. However, the BM aspiration and biopsy process is a very invasive and quite traumatic process for the

patients, which severely hinders the possibility of frequently monitoring of the disease.

3.2. MRD detection with liquid biopsy: concepts and modalities

As mentioned above, the requirement for a BM sample is a limitation of the current clinical MRD monitoring of AML. For instance, the small volume of collected BM aspirate may not reflect the potential involvement of the entire BM compartment. Therefore, this requires several BM samples to improve the accuracy of MDR determination.(Hong, Muller, Whiteside, et al., 2014) Nevertheless, for sampling reasons this is not possible. Therefore, it is highly desirable to find alternative methodologies to analyse MRD from samples of peripheral blood (PB) – the so-called "liquid biopsies". Indeed, as shown in figure 3, PB is an ideal alternative source of biological material to assess MRD since: i) it requires a minimally-invasive procedure to patients; ii) there is no limitation to the frequency of taking samples; and iii) sampling procedures are more cost-effective and technically facilitated. Altogether, the clinical use of validated PB liquid biopsies would allow a more frequent sampling and consequently a real-time monitoring of MRD over the entire course of AML management (Zeijlemaker et al., 2016).

Some studies were already performed aiming to compare the feasibility of using PB samples for MRD determination. Nevertheless, the results obtained so far indicate that when using liquid biopsies (PB samples) there is lower sensitivity than when using the standard BM specimens (Zeijlemaker et al., 2016; Jackson et al., 2016). In fact, AML is a disease which can manifest itself in an exuberant way in the PB, with leukaemic cells entering in circulation or, at least, releasing some leukaemic biomarkers such as cell-free nucleic acids (circulating DNA or RNA) and circulating extracellular vesicles (EVs) (Gold et al., 2015; Zhang et al., 2017). Nevertheless, the BM remains the site of origin of the AML, where most leukaemic blast cells reside. PB has the leukaemic components more diluted than the BM, thus sensitivity is a major problem when monitoring MRD in PB samples (Zeijlemaker et al., 2016).

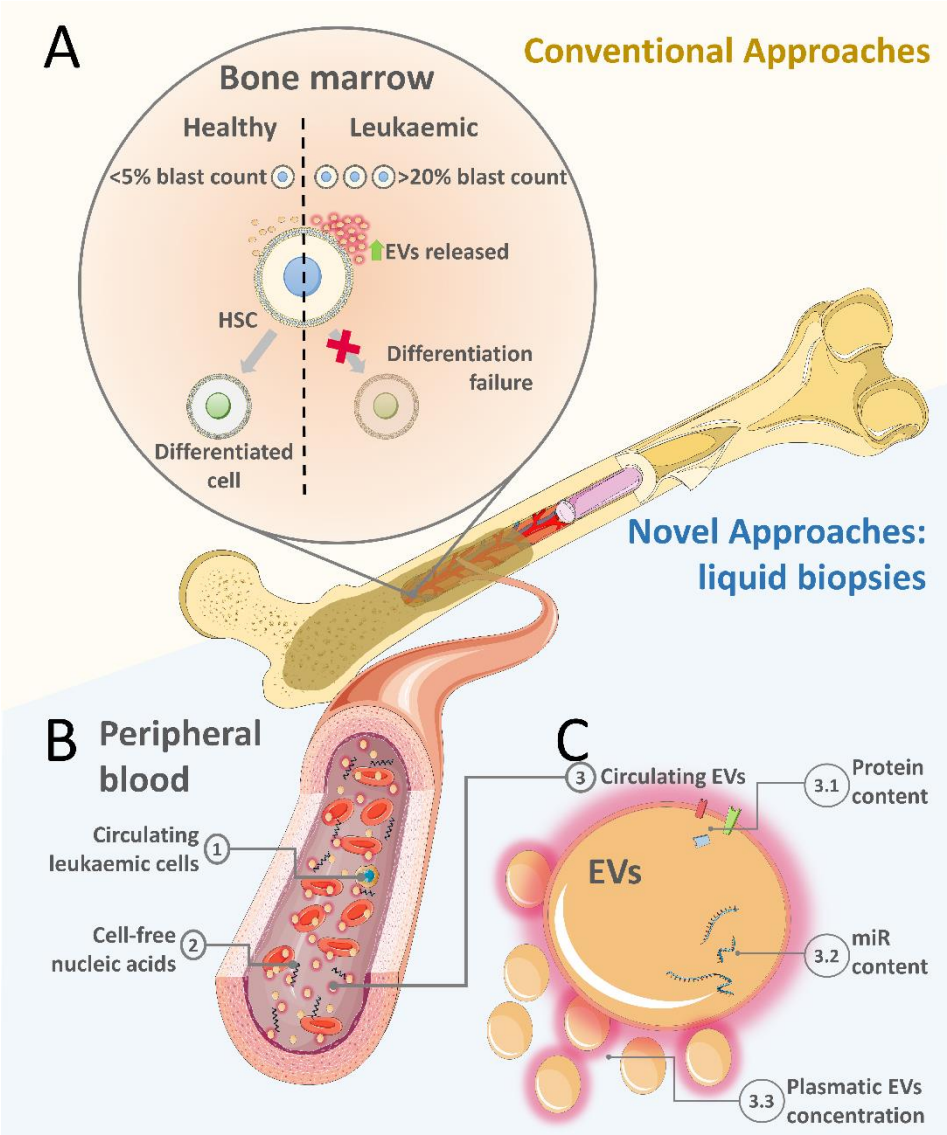

Figure 3 – Methodological approaches for Minimal Residual Disease (MRD) determination in AML. (A) During AML development, the leukaemic counterparts of haematopoietic stem cells (HSCs) gain proliferative advantage and lose their ability to differentiate, causing an abnormal accumulation of immature leukaemic blasts in the bone marrow (BM). In clinical routine, AML diagnosis is established based on the morphological analysis of the BM biopsy. WHO defined that AML diagnosis should be made when there is more than 20% of blasts in the BM aspirate sample. However, more than 5 % of blasts count is also indicative of blood disorder. Importantly, leukaemic cell transformation leads to an increase in extracellular vesicles (EVs) shedding by these blast cells. (B) The standard methodology to detect MRD is based on BM biopsy samples. Nevertheless, BM biopsy is a painful technique to the patients and requires specialized personnel and facilities, which altogether, severely limits the frequency of MRD monitoring. As a novel and emerging alternative, peripheral blood liquid-biopsies bypasses such restrains allowing real-time monitoring of the disease. MRD in peripheral blood-based liquid biopsies can be detected by 3 main approaches detection of circulating leukaemic cells (CLCs), analysis of circulating cancer cell nucleic acids and/or by profiling the circulating cancer cell-derived EVs. (C) EVs are small vesicles released from all cells that can be retrieved from virtually all biofluids such as blood and urine. These vesicles have the ability to carry, transport and protect biological information (AML biomarkers) from the donor leukaemic cells (e.g. proteins, lipids, nucleic acids). Importantly, since leukaemic cells have a drastic increase in EV shedding towards the blood stream, these vesicles have gained considerable interest as a reliable source of AML biomarkers. Indeed, profiling of AML patients' plasmatic EV concentration, together with their protein and nucleic acid content, could be used to improve the monitoring of AML disease.

This lack of sensitivity demands for an improvement on the methodologies used for PB analysis of MRD. One of the first approaches for MRD monitoring in PB resided in detecting the residual leukaemic cells. Alternative techniques focusing on other leukaemic biomarkers such as leukaemic cell-free nucleic acids or the cargo of circulating EVs derived from leukaemic cells are also being currently pursued. The feasibility of such approaches for MRD monitoring will be further addressed and their features discussed.

3.2.1. Circulating leukaemic cells

The analysis of circulating leukaemic cells (CLCs) is possible by collection and enrichment of these cells from blood. For that, techniques based on affinity for specific leukaemic antigens are used, relying on these antigens bond to magnetic beads which can then be isolated by magnetic-field cell sorting (Ao et al., 2016; Kallergi et al., 2016).

CLCs are excellent markers for MRD monitoring, since they carry the cancer-specific biomarkers. On the other hand, the extremely low number of leukaemic cells detected in low volumes of PB during "remission states" is a major issue. Therefore, the challenge to obtain high sensitivity resides on the lower number of CLCs obtained in low volumes of collected PB. These have been described to be as low as 1 CLC in 10^4-10^6 normal blood cells (Jackson et al., 2016). The ability to find CLCs from the blood, despite of the CLCs enrichment, is not good enough to be used as a stand-alone technique in the clinical practice.

3.2.2. Cell-free nucleic acids

The process involved in the isolation of cell-free nucleic acids is similar to the one involved in the isolation of CLCs. Briefly, a small amount of blood is collected and after that, the nucleic acids are isolated by affinity or precipitation methods. Monitoring nucleic acids such as DNA or miRs derived from cancer cells is a challenging task, given the several genetic mutations resulting from cancer development and the degradation of nucleic acids caused by enzymes present in the blood. Regarding the increase in the number of mutations, caused by the development of cancer and/or by therapeutic pressure, they may decrease the sensitivity of the follow-up when the aim is to detect a single and specific mutation

(Ma et al., 2015). Furthermore, the nucleic acids found in circulation are originated from virtually all cells of the organism, not only from the cancer cells. Some of the detected mutations could have origin in healthy cells without displaying any clinical significance but acting as an important confounding factor in the analysis. For higher sensitivity, digital PCR and next generation sequencing (NGS) are alternative approaches, which may be used to perform an accurate mutation profile of circulating nucleic acids (Zhang et al., 2017).

The digital PCR is a recent technology that identifies DNA mutations in a 100 000-fold excess of wildtype background (Hindson et al., 2011). NGS has an even higher sensitivity. NGS may detect most alterations in nucleic acids; however, there is a high-cost associated with this technique, together with a need for bioinformatic analysis of a massive amount of data. Unfortunately, such features are not compatible with the clinical practice in most hospitals (Chan et al., 2013; Zhang et al., 2017).

3.2.3. Circulating EVs released by leukaemic cells

EVs are released by all cell types, including by cancer cells. Interestingly, leukaemic-derived EVs can be found in the circulating PB in much higher concentration than the CLCs (Sadovska et al., 2015). These vesicles have an important particularity: they can be isolated from all biological fluids, including serum, plasma, saliva, urine, amniotic fluid, nasal and bronchial lavage fluid, breast milk and seminal fluid (Suchorska and Lach, 2016). Cells release a wide variety of EVs that could transport cargo from their donor cell. Proteins, lipids, enzymes and nucleic acids, are some of the examples of contents of the EV's cargo which may be transferred between different cells and influence the phenotype of the recipient cells. Importantly, the EV's cargo is enclosed by a lipid bilayer membrane which protects the cargo from degradation when in circulation (Tkach and Thery, 2016; Salomon et al., 2015).

The high EVs abundance in PB, together with their ability to transport and protect specific cargo derived from the donor cell and to carry specific proteins matching the donor cell on their surface are key features that allowed to increasingly recognized their potential as a source of biomarkers in a variety of diseases including cancer in general and AML in particular (Hong, Muller, Boyiadzis, et al., 2014; Boyiadzis and Whiteside, 2016; Tzoran et al., 2015).

3.2.3.1. EVs biogenesis

As mentioned above, EVs are membrane-contained vesicles that are released by cells. Regarding their biogenesis, there are 3 main types of EVs: (i) exosomes, (ii) microvesicles and (iii) apoptotic bodies.

The term "exosome" was initially wrongly used, to broadly characterize vesicles ranging from 30 to 1000 nm in size. Nevertheless, with increasing knowledge on the fundamental mechanisms of EVs biogenesis, there was a redefinition of the term "exosome" to refer to the smallest vesicles only, with a size range from 30 to 150 nm. The exosomes are originated on the endosomal system. During cells physiology, endosomes are matured and can eventually accumulate within intraluminal vesicles (ILVs), forming multi-vesicular bodies (MVBs). The inward budding of endosomal membrane generates these ILVs. During their formation, they selectively package some of the contents from the cell, such as proteins, nucleic acids and lipids (Klumperman and Raposo, 2014). Most MVBs are degraded by lysosomes. However, MVBs that express high levels of tetraspanins, and other molecules associated with the late endosomal pathway, can alternatively fuse with the cell membrane releasing their content. After the release of the ILVs to the extracellular milieu, these small vesicles are designated exosomes (Colombo et al., 2014).

Microvesicles, also known as ectosomes, have typical sizes from 70nm to 1000 nm. Therefore, the size of the smallest microvesicles may overlap with the exosomes size (Tkach and Thery, 2016). It is important to note that the exosome biogenesis differs from the microvesicles biogenesis. Microvesicles formation occurs by cytoskeletal protein contraction and membrane phospholipid redistribution (van der Pol et al., 2012). It is known that in living cells, the cell membrane is not uniform and is in constant movement, mainly due to translocases activity. It is believed that the translocation of phosphatidylserine to the outer-membrane leaflet may induce the budding development, and the contraction promoted by actin-myosin interactions completes the process of microvesicle formation (Akers et al., 2013).

The apoptotic bodies (AB) have a completely different biogenesis process, which is only activated during programmed cell death. Typically, these vesicles have a bigger size range when compared with the other EVs. Therefore, apoptotic bodies have a size range from 50 to 5000 nm. Although the specific mechanism for AB

formation remains unclear, they are probably derived from the membrane budding as a consequence of the actin-myosin interactions. An interesting characteristic of apoptotic bodies is the presence of cell organelles within these vesicles (Akers et al., 2013). Most apoptotic bodies are cleared locally by phagocytosis by macrophages. This process is based on the macrophage recognition of several markers expose in the cell membrane during apoptosis, as the phospholipid lysophosphatidylcholine (Erwig and Henson, 2008). Since the release of AB is intimately related with the programmed cell death process, for simplicity reasons and from here onwards the term "EVs" will be used to designate exosomes and microvesicles only.

3.2.3.2. EVs isolation

In the last couple of years, several alternative technical approaches were pursued to achieve the isolation of EVs from biological fluids. The most accepted EVs separation techniques include separation by density, by size or by the presence of specific protein markers at the EV's surface. All the possible EVs isolation methods have advantages and disadvantages, depending on the envisaged application. Indeed, different methods have distinct isolation efficiencies, may isolate distinct EV sub-populations, alter the isolated EVs functionality or even co-isolate plasma protein contaminants decreasing the isolated EV's purity. Therefore, the method of choice for plasmatic EV isolation for clinical applications is not a trivial choice. Thus, it is critical to choose the appropriate method for a given application, in order to not compromise the results and subsequentially the overall aim of the work. Here, some EV isolation methods will be described and compared and their feasibility to be implemented into a clinical setting will be discussed.

Density-based methods

The most widely used EVs isolation method is the ultracentrifugation, based on EV's density. Here, two modalities can be employed: the differential or the sucrose-gradient centrifugation.

The differential centrifugation consists of sequential steps of centrifugations resulting in a pellet of exosomes. Nevertheless, this technique may have an associated lack of reproducibility due to small variations in the protocol such as the speed of centrifugation, time of centrifugation, type of rotor or other factors

(Livshits et al., 2015). Other problems associated with this type of separation are related to the centrifuge forces required to form the EV pellet, which could damage the vesicles integrity, promoting their disruption and/or their aggregation or fusion. This could lead to decreased EV's marker detection and functionality. Moreover, with this isolation technique the EV's pellet will include most of the protein contaminants present in the original sample. This is a restrictive factor for clinical application, since it will severely affect the purity of the isolated EVs, given that biological samples are extremely rich in "contaminating" proteins (Webber and Clayton, 2013; Linares et al., 2015).

On the other hand, the sucrose-gradient ultracentrifugation is a method that combines the ultracentrifugation with a sucrose gradient. The density gradient allows separation of exosomes (with lower density) from microvesicles (with higher density). Indeed, this protocol allows obtaining EVs with higher purity than the differential ultracentrifugation protocol. However, this is more time-consuming, and also has the problem of low reproducibility and poor yield of EVs recovery (Taylor and Shah, 2015).

Moreover, the major issue related to these two protocols for clinical application is the requirement of an ultracentrifuge equipment at the hospital facilities. Moreover, the EVs isolation by ultracentrifugation is time-consumption which is prohibitive for a clinical setting.

Precipitation and affinity-based methods

In comparison with the ultracentrifugation-based methods, polymeric precipitation is a relatively fast technique that is based in the mixture of polymeric solutions that cause the precipitation of EVs. The major problem of such techniques is that they co-precipitate a high percentage of protein contaminants, such as lipoproteins, resulting in low purity of the isolated EVs when compared with other methods (Lobb et al., 2015). Another issue is that these methods may induce alterations on EVs, altering their morphology and affecting the expression of their protein markers. These reasons justify why this is not the method of election when studying the EVs physiology (Gamez-Valero et al., 2016).

Techniques based on EV protein affinity are also faster than ultracentrifugation and are more specific than polymeric precipitation. Indeed, the ability of separate the EVs by targeting known markers on their membrane surface allows the isolation of EVs with a high degree of purity. Usually the surface proteins targeted on EVs

for this type of isolation are the tetraspanins or immune-regulatory proteins. On the other hand, caution needs to be taken as this high specificity could be a disadvantage if one intents to study all EV sub-populations. Indeed, some important EV sub-populations may not express the used protein markers for EVs isolation (Oliveira-Rodriguez et al., 2016). Therefore, if the aim is to study a specific subpopulation of EVs, this technique guarantees high purity; however, if the aim is to study all EVs, this method is not adequate. Other important points are the elevated associated cost and the limitation to small amounts of sample.

Size-based methods

Size-exclusion chromatography (SEC) attracted a significant interest by the scientific community in the past few years as a methodology to isolate EVs from complex biological samples. SEC is a low cost technique, does not require large infrastructures and has high reproducibility since it is a simple single-step EV isolation method (Hong et al., 2016; Welton et al., 2015). This method requires a chromatography column filled with a compacted matrix composed of beads with specific-size pores. During sample elution, the vesicles may or not enter the pores of the beads, depending on their size. The result is the creation of a size-gradient where the vesicles are separated according to their sizes (Boing et al., 2014). Several studies comparing the benefits of the SEC method with other isolation methods have been made and SEC was categorized as a promising method for EV isolation in a clinical setting (Saenz-Cuesta et al., 2015; Mol et al., 2017; Xu et al., 2016). Beyond the simplicity and the low cost, this method allow obtaining undamaged EVs and with relatively low protein contaminants compared to the ultracentrifugation and precipitation methods (Muller et al., 2014; Saenz-Cuesta et al., 2015; Mol et al., 2017). This is a key point when functional EVs are necessary for downstream applications.

3.2.3.2.1. Distinguish EVs sub-types: a technically unsolved issue

As mentioned above, the EV isolation methodologies rely essentially in size and density principles. Technically, this becomes a serious drawback in the comprehension of exosomes and microvesicles biology, as it is challenging to distinguish between these two EV sub-types. Some researchers attempted to study these distinct EV subclasses. Most of them use these isolation methods and discuss

the results referring only to the enriched EV subtype. Nevertheless, the problem remains as the microvesicles and exosomes have an overlapping density and size range. In reality, these studies isolate a mixture of EVs of small and big sizes (Tkach and Thery, 2016; Raposo and Stoorvogel, 2013). This problem affected the inter-laboratory comparison of information about the EVs subclasses, making this area of research somewhat confusing.

Théry and her co-workers decided to tackle this issue, finding novel markers to distinguish the EVs as small-size-EVs and mid-size-EVs, disregarding their biogenesis. Adopting this perspective, they analysed the EVs subpopulations and detected protein markers that could separate small-EVs (CD81, syntenin-1, TSG101) from large-EVs (GP96, Actinin-4), and detected markers of EVs that are present in both subpopulations (HSP70, Flotilin-1, MHC I/II) (Kowal et al., 2016).

3.2.3.3. EVs as source of tumour biomarkers

In the last years, the scientific community developed an increasing interest in identifying protein tumour biomarkers present in the EVs isolated from biological fluids such as plasma (Fang et al., 2016; Horiguchi et al., 2016; Manri et al., 2017). Most interestingly, the concentration of plasmatic EVs may also become a useful indicator of the tumour burden. For instance, in prostate cancer patients the plasmatic EV concentration was higher when compared to healthy controls. Moreover, this observation was also valid for newly diagnosed AML patients whose serum contained higher levels of microvesicles when compared to healthy controls (Fais et al., 2016; Szczepanski et al., 2011).

Regarding the MRD monitoring by EV-based liquid biopsy, Tzoran *et al*. reported alterations on LAIPs expression in EVs during treatment. These authors observed a decrease in LAIPs up to normal levels in patients that achieved a CR clinical state, which correlated with an increase in overall survival (Tzoran et al., 2015). In addition to LAIPs, the same AML patients showed differences on the profile of TGF-β1 and Bcl-2 protein family in plasma exosomes. These authors suggested that such differences could reflect distinct responses to treatment (Hong, Muller, Whiteside, et al., 2014; Szczepanski et al., 2011; Wojtuszkiewicz et al., 2016). This is due to the ability of mature TGF-β1 to down-regulate NKG2D, an activating receptor present in natural-killer cells with a crucial role in immune response to cancer cells (Szczepanski et al., 2011; Nausch and Cerwenka, 2008).

Qualitative changes in Bcl-2 family may also serve as a predictor of drug resistance since these proteins are associated to apoptosis inhibition, resulting in a higher survival of the leukaemic cells (Wojtuszkiewicz et al., 2016).

Since EVs transport nucleic acids through the blood and protect them from degradation, they may contain mRNAs or miRs with genetic alterations on their cargo (Yu et al., 2016). Hornick *et al*. explored this possibility and attempted to measure the expression of a panel of miRs in exosomes isolated from the serum of AML patients, having obtained promising results (Hornick et al., 2015; Hornick et al., 2016). In the same line of thought, Caivano and their colleagues also started to study the presence of miR-155 in plasmatic EVs as a possible biomarker of haematological diseases (Caivano et al., 2016).

Despite these important ground-breaking works, the selection of a panel of proteins and miRs detected in the cargo of EVs from AML patients in order to detect MRD has not been done. Most importantly, their clinical validation requires analysis of many patients and healthy controls. For that, the standardization of the protocol for EVs isolation is mandatory and a careful use of EV nomenclature should be employed to avoid miscomprehension.

In conclusion, the low cost associated with EVs isolation and the easy access to liquid biopsies should allow frequent monitoring of MRD in AML patients. Therefore, if MRD assessment by analysis of circulating EVs becomes a clinical practice, it is possible that patient's relapse could be predicted sooner and with more accuracy, and the treatment provided sooner which would diminish disease progression and increase the patient's overall survival. Nevertheless, more studies are needed to establish an accurate biomarker signature on circulating EVs, able to measure MRD and better predict the prognosis of AML patients. This would allow to move the EV-based liquid biopsy concept from the bench towards the AML patients' bedside.

Scope of the thesis

1. Rationale & thesis aims

AML represents 12% of all haematological blood cancers (De Angelis et al., 2015). Although 80% of AML patients are able to achieve complete remission following induction chemotherapy, more than half will have disease recurrence. Importantly, only 5% of AML patients who are more than 65 years old are able to survive at 5 years after an AML diagnosis (Prada-Arismendy et al., 2016; Buccisano et al., 2012). In this rather poor clinical outcome, the detection of MRD allows the identification of patients who are at higher risk for relapse but also the assessment of chemotherapy response, providing critical information for therapy decision.

Currently, MRD assessment is performed by detection of remaining leukaemia cells within the patients' BM, which severely limits the frequency of disease monitoring (Hong, Muller, Boyiadzis, et al., 2014). Therefore, there is an increasing demand for alternative MRD detection methods on patients' PB for a faster, real-time and cost-effective monitoring of disease progression.

2. Hypothesis and goal

In this work, the idea that EV-based liquid biopsy can be used to detect MRD biomarkers in AML patients' PB was explored. To test this hypothesis, the main objective of this work was to establish a protocol for the isolation and characterization of the AML patients' circulating EVs. The specific aims of this thesis were as follows:

i. Implement a Size-Exclusion Chromatography (SEC) protocol for the isolation of circulating EVs from AML patients' blood samples

Bench-to-bedside implementation of EV-based liquid biopsies into the clinical practice can be compromised by the inappropriate isolation of plasma-derived EVs. The first aim was to implement and validate the use of SEC, a clinically compatible methodology, for the isolation of intact circulating EVs from AML patients' PB.

ii. Characterize the isolated EVs in the eluted SEC fractions

The validation of the SEC methodology may be performed by characterization of the isolated EVs, eluted in the distinct SEC fractions. The second aim was to characterize the nature of the eluted EVs in terms of EV morphology, size, particle concentration, protein concentration and presence of commonly-associated EV protein markers. Moreover, to analyse whether SEC elutes different EV sub-populations in the distinct fractions.

iii. Detect the presence of clinically established AML biomarkers in the patients' circulating EVs

To attest the feasibility of using EV-based liquid biopsies for MRD assessment in AML patients, the third aim was to confirm whether clinically established AML biomarkers are expressed in patients' circulating EVs.

iv. Evaluate the AML patients' EV profile during the course of treatment and clinical follow-up

The fourth aim was to understand whether the profile of circulating EVs is altered in AML patients and evaluate how these parameters change throughout treatment and clinical follow-up. For that, it was expected to isolate plasma-derived EVs from healthy controls and paired samples of AML patients at diagnosis, complete remission and at relapse.

It is expected that the work performed in this dissertation serves as a proof-of-principle for the feasibility of using EV-based liquid biopsy for MRD detection in AML patients, thus establishing the foundations for a future project in a larger AML patient cohort.

Material & Methods

1. Liquid biopsies from patients and healthy donors

1.1. Patient selection and blood collection

Human peripheral blood samples were collected from 4 AML patients admitted in the Clinical Haematology Service of "Centro Hospitalar de São João" (CHSJ) (Porto, Portugal), between February 2017 and July 2017. Paired samples of PB were collected from these patients at diagnosis and after achieving clinical complete remission upon the start of induction chemotherapy. All cases were new diagnosis and were submitted to an induction chemotherapy with high-dose ara-C treatment. Briefly, 6 mL of peripheral blood was collected to 3.5 mL tubes buffered with 3.8% of sodium citrate (0.129M) (Vacutest®, KIMA). Similarly, the human peripheral blood of 1 healthy volunteer with no known haematological malignancies was collected and used as healthy control. This study was approved by the ethical review board of "Centro Hospitalar de São João" and designed according to the tenets of the Declaration of Helsinki. All participants signed a written informed consent.

1.2. Clinical data collection

The clinical information from the AML patients was provided by the Clinical Haematology Service of CHSJ (Porto, Portugal), following patient anonymization. To anonymize each patient identity, it was generated an internal code number, which allowed sharing fully anonymized clinical information between the "Centro Hospitalar de São João" and the i3S laboratories. The clinical data shared by the Hospital included results from routine diagnostic exams from the patients at diagnosis, complete remission and relapse stages of the disease. In particular, the: (i) analytical analysis of the PB and the BM aspirates (hemograms and chemical analysis); (ii) analysis of patient's karyotype at the BM aspirates (cytogenetic analysis); (iii) identification and quantification of genetic mutations at the BM aspirates (molecular biology analysis); (iv) profiling of immune cells lineage/sub-populations at the BM aspirates by flow cytometry (immunophenotype analysis). During these routine medical exams, an extra 6 mL of PB was collected and transported to the i3S laboratory for EVs isolation and profiling.

Then, all the anonymized clinical data provided by "Centro Hospitalar de São João" was processed and integrated into individualized patient databases and

stored into an external hard-drive with limited access. This clinical data was correlated with some potential AML biomarkers found during the EVs profiling at the i3S laboratory.

1.3. Plasma isolation

The collected PB samples were maintained at room temperature (RT) in an orbital shaker at 1 g until processed for plasma isolation. For that, the blood samples were transferred to a 15 mL centrifuge tube and centrifuged at 2500 g (Megafuge 1.0R, Heraeus) for 15 min at 18-20ºC without brake. Upon initial centrifugation, three different layers were visible: erythrocytes and granulocytes (lower fraction); buffy coat, composed by leukocytes and most platelets (mid fraction); and platelets-rich plasma (upper fraction). The platelets-rich plasma layer was extracted into a 15 mL falcon tube and mixed with an equal volume of sterile PBS-Sodium Citrate at 0.32%. The mixture was then centrifuged at 2500 g (Megafuge 1.0R, Heraeus) for 15 min at 18-20 ºC and the supernatant containing poor-platelet plasma (PPP) was collected for another 15 mL falcon tube (Saenz-Cuesta et al., 2015). Aliquots of 1 mL of platelets-poor plasma were stored in 1.5 mL eppendorfs and frozen at -80 ºC until used for downstream analysis. From here onwards, this PPP will be mentioned only as blood plasma for simplicity reasons.

2. EVs isolation from plasma by size exclusion chromatography

The EVs isolation was performed by SEC using a method described by Anita Boing (Boing et al., 2014) with some modifications. Briefly, approximately 22 mL of Sepharose cross-linked 2B (CL2B300, Sigma) was washed with 0.32% Sodium Citrate in PBS and dropped on a 10 mL syringe (10 mL SOFT-JECT®, HSW), filled with a piece of stocking at the outlet. When the compacted sepharose reached the 10 mL mark, a 3MM paper filter was added on top to make an even stacking interface. Then, the column was submersed in 20% ethanol solution (bacteriostatic agent) and stored at 4 ºC until use.

Prior to EVs isolation, the SEC column was first washed with 0.32% Sodium Citrate in PBS (eluent) to remove the bacteriostatic agent. Then, before the eluent in the column dried-out, 1 mL of plasma was loaded and allowed to enter within the sepharose component before continuously adding more eluent for SEC isolation

of plasma-derived EVs. A total of 10 fractions with 1 mL each were collected in 1.5 mL eppendorfs and stored temporarily at 4 ºC. Then, some of the collected fractions proceeded directly for EV's characterization methodologies and/or were stored at -80 ºC until further use.

After EVs isolation, the SEC columns were washed twice with eluent and one more time with a solution of 0.01% Triton in PBS (to make sure that all sample remnants were eluted from the column). Finally, all the Triton-PBS solution was removed with eluent and replaced with bacteriostatic agent, prior to storage of the column at 4 ºC.

3. EVs characterization

3.1. Dynamic light scattering

Dynamic light scattering (DLS) was used to profile the size of the EVs eluted from each SEC fraction. For that, 90 µL of each fraction was added to a polystyrene cuvette (ZEN0040, Malvern) with a 10 mm path length for DLS analysis with a Zetasizer Nano ZS system (Malvern Instruments) as previously described (Lopes-Rodrigues et al., 2016). DLS data was acquired and analysed with the Zetasizer Software version 7.12. Briefly, DLS measurements were performed at 25 ºC following the incidence of a light beam at 633nm in the cuvette. The back light scattered was recorded at an angle of 173º. The obtained DLS data is based on the Stokes-Einstein equation. Therefore, for a more accurate result DLS recordings were mathematically corrected with an EV's refractive index of 1.39 and absorption index of 0.01, as reported by others (van der Pol et al., 2014; Gardiner et al., 2014; Valkonen et al., 2017). The dispersant viscosity was considered as 0.89 cP and the refractive index considered as 1.33. The DLS data was plotted as particle number (%) versus particle size (nm).

3.2. Nanoparticle tracking analysis

The particle size was estimated by nanoparticle tracking analysis (NTA). The NTA also allowed quantification of the particle concentration in each SEC fraction. The EVs from SEC fractions 3 to 7 were separately vortexed and immediately loaded in a NanoSight NS300 (Malvern Instruments) with a 1 mL syringe (Omnifix® 100 Solo, B|BRAUM). For sample loading, a NanoSight syringe pump (Malvern

Instruments) was used to infuse the samples at constant flow rate into the NanoSight NS300 (Malvern Instruments) at RT. Then, three separate 30-seconds videos were recorded, with the following specifications: Camara Type sCMOS; Laser Type Blue488; Camera Level 15; Slider Shutter 1206; Slider Gain 366; FPS 25.0; Temperature 24.0 – 24.0 ºC; Viscosity 0.909 – 0.910 cP; Syringe Pump Speed 40. Particles were detected by video analysis using a NanoSight NTA Software version (NTA 3.2 Dev Build 3.2.16) with the following settings: Detection Threshold 5; Blur Size Auto; and Max Jump Distance Auto (8.3 – 16.2 pixels).

The NTA equipment provides optimal measurements for sample concentrations in the range of 10^7 to 10^9 particles/mL. Considering this, prior to EVs loading, SEC fraction 3 and SEC fractions 4 to 7 were pre-diluted in 0.32% Sodium Citrate in PBS at 1:100 and 1:1000, respectively. The NTA data was plotted as particle concentration per SEC fraction.

3.3. Transmission electron microscopy

Negative staining transmission electron microscopy (TEM) was used for the morphological assessment of isolated EVs, as previously described (Lopes-Rodrigues et al., 2016). Briefly, 12 µL of freshly isolated EVs from SEC fractions 3 to 7 were separately mounted in Formvar-Carbon coated electron microscopy grids (FCF300-Ni, EMS) for 2 min and dried with a filter paper. Then, TEM grids were counter-stained with uranyl acetate and observed with a Jeol JEM 1400 at 80 keV and with 10^5 times amplifications. Fractions 5 to 7 were pre-diluted 1:2 with 0.32% Sodium Citrate in PBS prior to loading in TEM grids for a proper EV visualization. Data was obtained by the Histology and Electron Microscopy Service, i3S, Porto.

Representative TEM photographs were acquired and the EV size for each individual SEC fraction was measured by image analysis using the ImageJ software. A minimum of 200 EVs were counted for each SEC fraction. Size profile of EVs from SEC fractions 4 to 7 was plotted as frequency curves.

4. EVs content analysis

4.1. Protein analysis by Western blot

4.1.1. Protein quantification

The amount of protein in each SEC fraction (either EV-bounded or plasma protein contaminants) was quantified in a microplate with the Lowry-based protein detection method, accordingly to the manufacturer protocol (DC™ Protein assay, Bio-Rad). After a minimum of 30 min incubation in a dark chamber, microplate well absorbance was analysed in a microplate reader (Synergy™ Mx, BioTek) with a 488 nm excitation wavelength and read at the 655 nm emission. The data was obtained with the Gen5 Software and exported to the Microsoft Excel 2016 Software for further analysis. Data was plotted as protein concentration (µg/mL) per SEC fraction.

4.1.2. Sample concentration

SEC fractions 3 to 6 were concentred up to 10-fold by Speed Vacuum (SPD121P Digital Series SpeedVac™, SystemsThermo Fisher Scientific™). The procedure was performed at RT with the vacuum pressure set on 1.2 torr. Concentrated SEC samples were individually quantified or pooled together for protein quantification, as previously described.

4.1.3. Protein separation and transfer to a membrane

The isolated EVs were first lysed by adding loading buffer (Tris-HCl 1M pH 6.8, 10% SDS, 85% glicerol, β-mercaptoethanol, 1% bromophenol blue) and boiling at 95 ºC for 5 minutes. Then, a similar amount of protein for each condition was loaded into each well of either 8% or 12% sodium dodecyl sulfate polyacrylamide gels for electrophoresis (SDS-PAGE), depending on the molecular weight (MW) of the protein of interest. The gels were composed by an initial stacking gel (Tris-HCl 1.0 M at pH 6.8, 30% acrylamide, 10% SDS, 10% ammonium persulfate, TEMED) and a resolving gel (Tris-HCl 1.0 M at pH 8.8, 30% acrylamide, 10% SDS, 10% ammonium persulfate, TEMED) with the intended acrylamide final percentage. The EV-derived proteins were then separated by size by SDS-PAGE. The electrophoresis started at 70 V while the samples went through the stacking gel and the voltage was increased to 100 V when samples were in the resolving gel. The SDS-PAGE

technique was performed in a Mini-PROTEAN® Tetra Vertical Electrophoresis Cell system (Bio-Rad), filled with running buffer prepared by diluting 1:10 the 10X TGS solution (Bio-Rad) in 900 mL of deionized water. The separated proteins on the gel were then transferred to a nitrocellulose membrane (Amersham Protran 0.45 NC, GE Healthcare) by using a Mini Trans-Blot® Cell system (Bio-Rad) with transfer buffer prepared by diluting 1:10 10X TG solution (Bio-Rad) in 700 mL of deionised water and 200 mL of methanol. The transfer was carried out at 100 V for 2 hours.

4.1.4. Protein detection

Membranes were blocked for 2 h in freshly prepared 5% milk in tris-buffered saline solution with 0.1% Tween-20 (Promega), with agitation at 1 g (Standard Orbital Shaker 1000, VWR). After blocking the nitrocellulose membranes, they were incubated with primary antibodies in a 15 mL or 50 mL Falcon for 1,5 h using a roller tube shaker (Roller Mixer SRT9D, STUART®) at RT. The following primary antibodies were used for Western blotting: anti-Actinin-4 (1:200, GeneTex, GTX113116), anti-annexin XI (1:100, Santa Cruz Biotechnology, sc-271487), anti-Albumin (1:2000, Santa Cruz Biotechnology, sc-271605), anti-CD33 (1:200, abcam, ab194662), anti-CD34 (1:10000, abcam, ab81289), anti-CD63 (1:1000, SBI, EXOAB-CD63A-1), anti-CD117 (1:200, abcam, ab5505), anti-CD123 (1:200, Santa Cruz Biotechnology, sc-455), anti-Cytochrome C (1:1000, Santa Cruz Biotechnology, sc-13560), anti-FLT3 (1:100, Santa Cruz Biotechnology, sc-101343), anti-HSP70 (1:500, SBI, EXOAB-Hsp70A-1), anti-mitofilin (1:100, Santa Cruz Biotechnology, sc-390706), anti-myeloperoxidase (1:100, Santa Cruz Biotechnology, sc-66107), and anti-nucleophosmin (1:100, Santa Cruz Biotechnologies, sc-271737). Depending on the species reactivity of the used primary antibody, anti-mouse IgG-HRP (1:2000; Santa Cruz Biotechnology, sc-2031), anti-rabbit IgG-HRP (1:2000; Santa Cruz Biotechnology, sc-2004) or anti-goat IgG-HRP (1:2000; Santa Cruz Biotechnology, sc-2020) secondary antibodies were incubated for 1 h, in conditions similar to the incubations of the primary antibodies. The nitrocellulose membranes were washed 3 times (during 10 min/wash) with tris-buffered saline solution with 0.1% Tween-20 before and after the incubation with secondary antibodies.

Protein bands were detected in the nitrocellulose membranes by chemiluminescence. For that, nitrocellulose membranes were incubated with a chemiluminescence solution (ECL Western Blotting Detection Reagent, GE

Healthcare) and, in a dark room, a chemiluminescence film (Amersham Hyperfilm™ ECL, GE Healthcare) was placed on top of membranes. The signal from the protein bands is then transmitted from the nitrocellulose membranes to the chemiluminescence films. Signal was detected using commercially available developer and fixer solutions (Kodak GBX, Sigma).

4.2. miRs analysis by real time qRT-PCR

4.2.1. RNA extraction

The RNA extraction from SEC fractions was performed with a miRCURY™ RNA isolation kit (Biofluids, EXIQON). Prior to RNA extraction, the SEC fractions 3 to 7 were pooled together and pre-treated with proteinase K (Thermo Scientific) accordingly to the miRCURY™ RNA isolation kit protocol. Briefly, 900 µL of pooled EVs isolated by SEC were lysed and the protein precipitated with kit solutions. Then, the samples were centrifuged at 11000 g (Micro Star 17R, VWR) for 3 min and the supernatant was loaded into a microRNA Mini Spin Column BF. The total RNA was bond to the column during a 2-min incubation and the contaminants were removed with the kit wash solutions. The total RNA was eluted from the column with RNase free water by centrifugation at 11000 g (Micro Star 17R, VWR) for 1 min.

Total RNA quantification was performed using a NanoDrop™ 1000 Spectophotometer (Thermo Scientific). All the purified RNA samples proceeded directly for cDNA synthesis or stored at -80 ºC until further use.

4.2.2. cDNA synthesis by reverse transcription

cDNA synthesis from the isolated EV-derived RNAs was performed by reverse transcription using the miScript II RT Kit (QIAGEN), accordingly to the manufacturer instructions. Briefly, a master mix for reverse-transcription was composed by 4 µL of 5x HiSpec Buffer, 2 µL of 10x miScript Nucleics Mix and 2 µL of miScript Reverse Transcriptase Mix. Each PCR tube was filled with variable RNAse free water and template RNA volumes to obtain a final reaction volume of 20 µL. PCR tubes with no sample or without the enzyme were used as negative controls. Tubes were incubated for 60 min at 37 ºC following an additional 5 min at 95 ºC to inactivate the reverse transcriptase enzyme. Samples were then hold at 4 ºC in a thermocycler

(MyCycler™ Personal Thermal Cycler, Bio-Rad) or stored at -20 ºC until further analysis.

4.2.3. Real time quantitative PCR

EV-derived miRs were profiled by qRT-PCR using the SYBR Green PCR Master Mix (QIAGEN), accordingly to the manufacturer instructions. Briefly, a 96-wells plate was used to perform the qRT-PCR, where each well contained 10 µL of 2x QuantiTect SYBR Green PCR Master Mix, 2 µL of 10x miScript Universal Primer, 2 µL of 10x miScript Primer assay (miR-16, miR-150 or miR-155), 5 µL of RNase free water and 1 µL of template cDNA. Duplicates for each RNA sample were used. Additionally, individual standard curves were prepared for each miR primer assay. The standard curves were made by using serial dilutions of cDNA prepared from the HL-60 cell line. cDNA sample was replaced by RNase free-water in the negative controls. Samples were spun in the well-plate using a centrifuge (centrifuge 5810 R, eppendorf). Then, the template and the cycling program were planed using the 7500 Software v2.0.6 (Applied Biosystems). PCR amplification was performed in a 7500 Fast Real-Time PCR System (Applied Biosystems) set with the following program: 95ºC for 15 min for enzyme activation, followed by 40 cycles for denaturation of samples at 94ºC during 15 sec, annealing at 55ºC for 30 sec and extension at 70 ºC for 30 sec. The results were analysed using the above mentioned 7500 Software v2.0.6 (Applied Biosystems).

5. Statistical analysis

Data were summarized by descriptive statistics: (i) means and standard errors of the means for symmetric distributions in continued variables; (ii) medians and interquartile amplitudes for asymmetric distributions in continued variables and (iii) frequencies and percentages for categorical variables. For data normally distributed, the statistical analyses were performed using paired and unpaired two-tailed Student's t-test. When data was not normally distributed, the Wilcoxon-Mann-Whitney test was performed. To consider a statistical significant data, a p value <0.05 was established.

Results

1. Intact and size-resolved EVs were isolated by Size Exclusion Chromatography from the peripheral blood of AML patients

Circulating EVs have been increasingly recognized as a potential source of oncologic disease biomarkers, particularly in tumours of haematological origin, as AML. Several options are currently available to isolate these plasma-derived EVs from the patients' peripheral blood. The most widely used technique for EV isolation consists on ultracentrifugation density-based methods. Nevertheless, this is a time-consuming methodology and presents important drawbacks for bench-to-bedside implementation of EV-based liquid biopsies into the clinical practice. Here, an alternative and compatible protocol for implementation into a clinical setting for the isolation of plasma-derived EVs was explored.

AML patients' peripheral blood was collected, briefly centrifuged to obtain PPP and diluted in an equal volume of PBS-Sodium Citrate at 0.32%. Then, 1 mL of this pre-diluted PPP was applied into a home-made SEC column as described by others (Boing et al., 2014) with some modifications, to isolate plasma-derived EVs. Upon SEC, the PPP constituents were separated into 10 individual SEC fractions of 1 mL each.

To confirm the successful isolation of plasma-derived EVs in the collected SEC fractions, the particle size, concentration, morphology and protein content was investigated **(Figure 4)**.

DLS analysis on the collected SEC fractions reveals that PPP constituents (mostly EVs – including apoptotic bodies, microvesicles, exosomes – but also a wide range of plasma proteins) have a size ranging from 4 to 1000 nm. Importantly, upon SEC, these PPP constituents were resolved by size and individualized throughout the 10 eluted SEC fractions **(Figure 4A)**. Indeed, the isolated PPP constituents with a higher size (most likely apoptotic bodies and microvesicles) are eluted first while the PPP constituents with a smaller size (eventually exosomes and plasma proteins) are eluted in the later fractions. Accordingly, the PPP constituents eluted in fraction F1 had a mean particle size of 599,8 ± 114,6 nm, followed by F2 with 478,4 ± 105,5 nm, F3 with 128,3 ± 120,7 nm, F4 with 83,95 ± 48,92 nm, F5 with 47,49 ± 16,89 nm, F6 with 23,56 ± 7,61 nm, F7 with 13,30 ± 4,84 nm, F8 with 6,72 ± 2,17 nm, F9 with 6,31 ± 1,96 nm and finally F10 with 6,30 ± 1,87 nm.

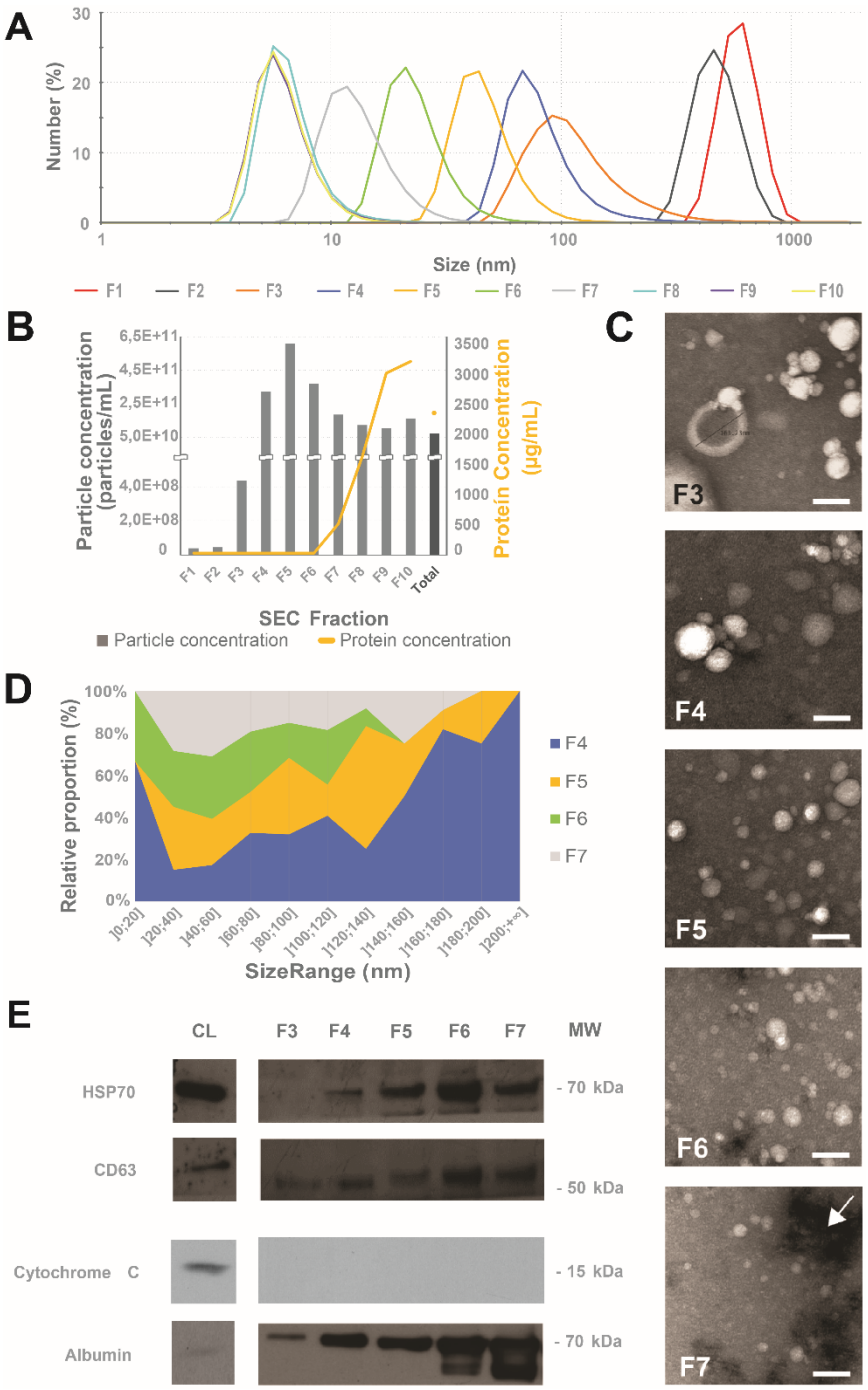

Figure 4 – Size exclusion chromatography (SEC) allows the isolation of intact EVs, resolved by size, from the blood plasma of AML patients. (A) Size percentage of EVs in each SEC fraction isolated from AML patient's blood plasma, using dynamic light scattering (DLS). (B) EVs concentration (grey bars; left axis) plotted against protein concentration (yellow line; right axis) measured by nanoparticle tracking analysis (NTA) and Lowry method respectively, for each SEC fraction. (C) Representative TEM photographs of EVs morphology collected from SEC fractions F3, F4, F5, F6 and F7. EVs collected from fraction 7 onwards were eluted with some protein "contaminants" from plasma (arrowed). Fractions 5, 6 and 7 were diluted 2-fold in 0.32% Sodium Citrate in PBS prior to imaging. Scale bar: 100 nm. (D) Representative proportion of resolved EVs size distribution profile in each SEC fraction. A minimum of 200 EVs were measured on transmission electron microscopy (TEM) images for each individual SEC fraction, using the ImageJ software. EVs collected in fraction 3 were found at sparse number in TEM photographs and therefore were not eligible for manual EV count. (E) Presence of established EV protein markers (HSP70 and CD63) and other "contaminants" (Cytochrome C and Albumin) in the SEC fractions. CL: HL60 cell lysate. The gels loading was confirmed by Ponceau (Supplementary figure 1).

While performing particle size characterization of the collected SEC fractions, the corresponding particle and total protein concentration were also quantified by nanoparticle tracking analysis and Lowry methods, respectively **(Figure 4B)**.

The collected SEC fractions had a mean particle concentration ranging from $3,8x10^{7}$ to $6x10^{11}$particles per mL. Indeed, fractions F1 to F3 had a low but increasing particle concentration ($3,8x10^{7}$, $4,4x10^{7}$ and $4,3x10^{8}$ particles/mL, respectively). Importantly, the following fractions had a drastic increase in particle concentration. The concentration of fraction F4 was in the order of 10^{11}, which was maintained high up to fraction 10, peaking at fraction 5 with $6,1x10^{11}$ particles/mL. Overall, SEC allowed isolation of an average of $1,44x10^{12}$ particles per mL of PPP. Additionally, total protein quantification revealed that fractions F1 to F6 had no protein (or below detection limit), while increasing protein concentrations were eluted in the following fractions. Indeed, protein was initially detected in fraction 7 (489,5 µg/mL) and the following fractions had an exponential increase in the protein concentration, peaking at fraction 10 (3197 µg/mL) (**Figure 4B**).

TEM imaging revealed that the isolated PPP constituents in the SEC fractions F3 to F7 resembled the lipid nature of EVs. The imaged particles were characterized by an intact round shape with a mean diameter ranging from 20-200 nm which is consistent with the isolation of undamaged plasma-derived EVs (**Figure 4C**). Noteworthy, the low particle concentration did not allow TEM imaging in fractions F1 and F2 while high amounts of protein aggregates (depicted as black areas) hampered the particle visualization in fractions F8 to F10. Additionally, TEM images showed that the small EVs isolated in fractions F6 and F7 were co-eluted with low amounts of contaminating plasma protein aggregates (**Figure 4C**, depicted as a white arrow in fraction F7), thus corroborating the data obtained by NTA and protein quantification.

Consistently with DLS and NTA data, the size measurement of more than 200 EVs per SEC fraction, in the TEM photographs, confirmed that early eluted fractions had a higher proportion of larger vesicles while later fractions were predominantly composed by smaller vesicles and protein aggregates (**Figure 4D**).

To further confirm that the PPP constituents isolated from fractions F3 to F7 were EVs, the presence of established EV protein markers in these SEC fractions was evaluated by WB analysis (**Figure 4E**). Noteworthy, since these SEC fractions presented non-detectable or very low concentrations of protein, prior EV

concentration by speed vacuum was required in order to isolate quantifiable amounts of protein and perform the WB technique. Importantly, the microvesicle/exosome nature of isolated PPP constituents from fractions F3 to F7 was confirmed by the presence of the tetraspanins family protein CD63 and the cytosolic protein HSP70, both considered to be EV's markers. Notably, the absence of a cell organelle marker, cytochrome c, in the isolated fractions further confirmed that no cell debris or apoptotic body contaminants were co-eluted with plasma-derived EVs in these fractions (F3 to F7).

Albumin is one of the most abundant plasma protein "contaminants", which may hamper the isolation of plasma-derived EVs. Therefore, the purity of isolated EVs was further evaluated by analysing albumin expression in fractions F3 to F7. Unfortunately, all fractions seemed to co-elute albumin together with the plasma-derived EVs, although at very distinct levels (**Figure 4E**). Indeed, later fractions presented higher amounts of albumin, corroborating the protein quantification and TEM imaging data previously obtained which showed that fraction 7 and the following ones co-eluted small EVs together with "protein contaminants".

2. Recently reported size-dependent EV markers were differently expressed in the eluted SEC fractions

Physiologically, all cells exhibit an heterogeneous release of EVs of different sizes. Most notably, these distinct EV's sub-populations seem to carry different cargos, serving most likely different purposes (Raposo and Stoorvogel, 2013). Importantly, the EVs biogenesis seems to be consistently altered by the carcinogenic transformation events. Indeed, several groups (Manri et al., 2017; Sadovska et al., 2015; Salomon et al., 2015), including our own (Lopes-Rodrigues et al., 2016), reported that the type of EV sub-populations shed by the tumour cells could serve as a predictive biomarker of the tumour cell phenotype.

Here this work sought to understand whether the recently reported size-markers (Kowal et al., 2016) for small (annexin XI) and mid-size (actinin-4 and mitofilin) EVs were differently enriched throughout the various collected SEC fractions. Results (WB analysis) revealed that the mid-size EV marker actinin-4 is the only marker present in fraction 3 (**Figure 5**). Importantly, actinin-4 abundance was higher at fraction 4, decreasing its expression from fraction F5 onwards. On the other hand, mitofilin was not detected in fraction F3 and was scarcely expressed

in fraction F4. Mitofilin was only properly detected in fraction 5 and its expression increased in the subsequent analysed fractions (F6 and F7). Regarding the small EV marker, annexin XI, it is only highly expressed at the later fractions F6 and F7 (**Figure 5**).

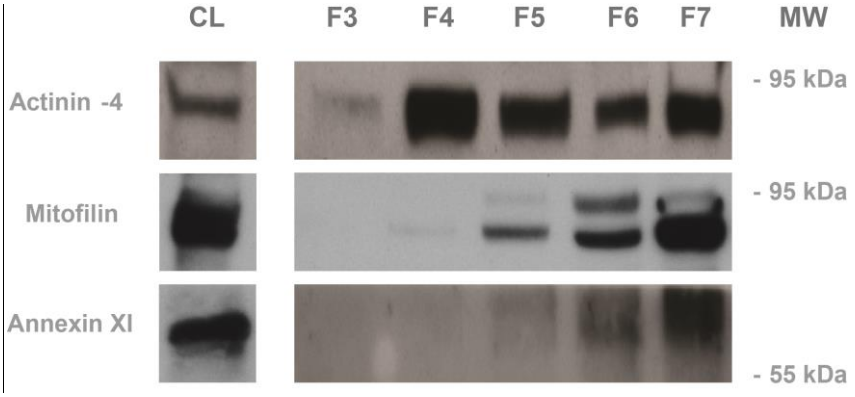

Figure 5 - Isolation of different size-resolved EV sub-populations by size exclusion chromatography. Analysis of mid-size EV markers (actinin-4 and mitofilin) and small-size EV markers (annexin XI) distribution profile in fractions 3 to 7. CL: HL60 cell lysate. The gels loading was confirmed by Ponceau (Supplementary figure 2).

Therefore, plasma-derived EVs can be broadly separated by SEC into mid-size EVs (typically EVs that often originate from the budding of the cell's plasma membrane) and small-size EVs (usually enriched in small exosomes formed by the endosomal compartment).

3. Patients' clinical information

The AML samples were collected from 4 AML patients admitted in the Clinical Haematology Service of "Centro Hospitalar de São João". The relevant AML patients' clinical information **was obtained from CHSJ** and is depicted in **Table 1**.

3.1. Patient #1_AML

Patient 1 (#1_AML) was male and 33 years old. The abnormalities in the blood counts of this patient were the reason for the doctors request for a BM biopsy. Immune-phenotype analysis detected the presence of proteins associated to AML. Clinically, this patient was stratified as a poor prognosis AML due to the presence of a cytogenetic deletion in chromosome 9 and a FLT3-ITD+ mutation. At Day 30 (D30) of treatment with high dose induction chemotherapy with ara-C, BM biopsy revealed a normal karyotype and low quantity of blasts. The patient was clinically

Table 1 – Clinical data provided by the Clinical Haematology Service of Centro Hospitalar de São João (CHS-J). Clinical data from four AML patients at diagnosis, following complete remission and at relapse (when applicable), including clinical data collected by CHS-JJ on patient's peripheral blood cell counts and chemical analysis, BM immunophenotyping, karyotype analysis and molecular profile of FLT3, NMP1 and RUNX mutations.

# ID	Sex	Age	AML Status	Blood Counts				Immuno-phenotype	Karyotype (0/20: karyotype)	Molecular Profile FLT3-ITD FLT3 TKD D835 NMP1 (exon 12) RUNX1-RUNX1T1
				Leukocytes (x10^9/L)	DHL/LDH (U/L)	Total protein (g/L)	Albumin (g/L)			
#1_AML	M	33	Active	90,89	2008	61,8	31,5	CD13+; CD33+; CD34+ (32,5%); CD64+; CD117+; CD123+; HLA-DR+; MPO+	16: 46,XY,del(9)(q11q32) 4: 46,XY	FLT3-ITD+
			Remission	6,1	206	68,1	35,3	CD34+ (2,2%) CD34+/CD13+ (2,0%) CD117+ (2,5%) CD34+/CD117+/HLA-DR+ (1,7%)	20: 46,XY	Negative
			Relapse	1,75	N/A	N/A	N/A	CD34+ (39%) CD34+/CD13+ (37,2%) CD117+ (33,4%) CD34+/CD117+/HLA-DR+ (32,2%)	3: 46,XY,del(9)(q11q32) 17: 46,XY	FLT3-ITD+
#2_AML	F	34	Active	0,6	301	N/A	37,0	CD7+ (52%); CD13+; CD33het+; CD34+ (65%); CD117+; CD123+; HLA-DR+ (90%); MPO+	2: 48,XX,del(3)(p13),-5,-17,-17,+19,+22,+22,+2mar 13: 46,XX,del(3)(p13),-5,-17,-17,+22,+2mar 4: 47,XX,del(3)(p13),-5,-17,+22,+2mar 1: 46,XX	Negative
			Remission	4,34	282	67,8	37,5	CD34+ (1,5%) CD34+/CD13+ (1,4%) CD117+ (3,9%) CD34+/CD117+/HLA-DR+ (0,3%)	20: 46,XX	Negative
#3_AML	M	36	Active	8,53	489	N/A	44,1	CD13+; CD33+; CD34+ (70%); CD56+ (21%); CD64+; CD117+; CD123+; HLA-DR+; MPO+	20: 45,X,-Y,t(8,21)(q22;q22)	RUNX1-RUNX1T1+
			Remission	5,47	145	67,2	39,2	CD34+ (1,3%) CD34+/CD13+ (1,0%) CD117+ (2,6%) CD34+/CD117+/HLA-DR+ (0,8%)	20: 46,XY	RUNX1-RUNX1T1+
#4_AML	M	70	Active	82,43	638	59,2	26,9	CD7+(56%); CD13+(85%); CD33+; CD38+; CD56+(39%); CD64het+; CD117+; CD123+; HLA-DR+; MPO+	20: 46,XY	NPM1+

considered to be in a complete remission. After a consolidation therapy, the patient had a relapse and was re-induced with FLAG-Ida (combination of Fludarabine, ara-C and Idarubicin) + Sorafenib and another cycle of Azacytidine + Sorafanib. The patient achieved a partial remission and will be submitted to allogeneic haematopoietic stem cell transplantation in "Instituto Português de Oncologia do Porto Francisco Gentil" (IPO), Porto, Portugal.

3.2. Patient #2_AML

The patient 2 (#2_AML), a 34 years old female, arrived at CHSJ with a suspicion of AML which was validated by a BM biopsy. This patient presented a high level of AML markers in the immune-phenotype analysis with a complex karyotype and was clinically stratified as a poor prognosis AML (**Table 1**). This patient achieved complete remission after a high-dose chemotherapy cycle which was maintained until the time of writing this thesis. BM biopsy was performed to monitor the disease at Day 30 (D30) post-induction and was MRD negative. Despite this, the complex karyotype of the disease is strongly associated with a high risk for relapse. Therefore, to prevent this scenario, allogeneic haematopoietic stem cell transplantation in IPO was preventively offered to the patient.

3.3. Patient #3_AML

The third patient (#3_AML) was a 36 years old male, diagnosed with AML which was stratified as a favourable prognosis AML. This stratification was justified by the presence of a translocation (8,21) and gene fusion RUNX1-RUNX1T1 without the FLT3-ITD+ mutation. The patient achieved complete remission after a high-dose induction chemotherapy cycle. A low level of RUNX1-RUNX1T1 biomarker was maintained at CR. The patient was then submitted to another 3 cycles of consolidation chemotherapy.

3.4. Patient #4_AML

Patient 4 (#4_AML) was a 70 years old male diagnosed with AML. BM biopsy was performed and revealed the presence of elevated levels of AML markers in immune-phenotype analysis, but a normal karyotype. Additionally, molecular analysis revealed a mutation in exon 12 of NPM1 gene. The patient was clinically stratified as a favourable prognosis AML and submitted to induction chemotherapy within a clinical trial. The patient was unable to achieve complete remission and died at D31 post-induction therapy.

4. AML patients presented a higher concentration of plasmatic EVs at complete remission

The plasmatic concentration of circulating EVs in cancer patients has been reported as a potential predictive biomarker of the disease stage (Fais et al., 2016). Considering this, here it was sought to understand whether there were differences on the plasmatic concentration and size profile of AML patients circulating EVs, over the course of AML evolution.

Preliminary results showed that NTA measurements revealed that for most AML patients the plasmatic EV concentration is slightly lower when compared with a healthy control, with the exception of patient #3_AML (**Figure 6A, Table 2**). Importantly, in the four patients analysed, was found that the plasmatic EV concentration was drastically increased at complete remission. Noteworthy, the AML relapse in patient #1_AML was also characterized by an increase of circulating EVs. No correlation was found between the total protein amount isolated in the pooled SEC fractions and patients' clinical state (**Figure 6A**).

Table 2 – Summary of size and concentration of plasmatic EVs isolated from AML patient's plasma. EV mode size and plasmatic EV concentration were measured by NTA in samples collected at diagnosis (D), complete remission (R) and relapse from four AML patients and one healthy control (H). Regarding EVs concentration, the ratio of R/D and of D/H were analysed. ID of AML patients: #1_AML, #2_AML, #3_AML and #4_AML.

# ID	Prognosis	AML Status	Size Mode (nm)	Plasmatic EV Concentration		
				Evs/mL	Ratio R/D (Folds)	Ratio D/H (Folds)
#1_AML	Poor	Diagnosis	74	7,19E+10	7,3	0,9
		Remission	75	5,22E+11		
		Relapse	77,3	1,83E+11	N/A	
#2_AML	Poor	Diagnosis	78,8	5,64E+10	4,0	0,7
		Remission	87,1	2,23E+11		
#3_AML	Favourable	Diagnosis	66,7	1,81E+11	1,0	2,1
		Remission	64	1,91E+11		
#4_AML	Favourable	Diagnosis	66,5	6,22E+10	N/A	0,8
Healthy control	N/A	Healthy	63	8,29E+10	N/A	N/A

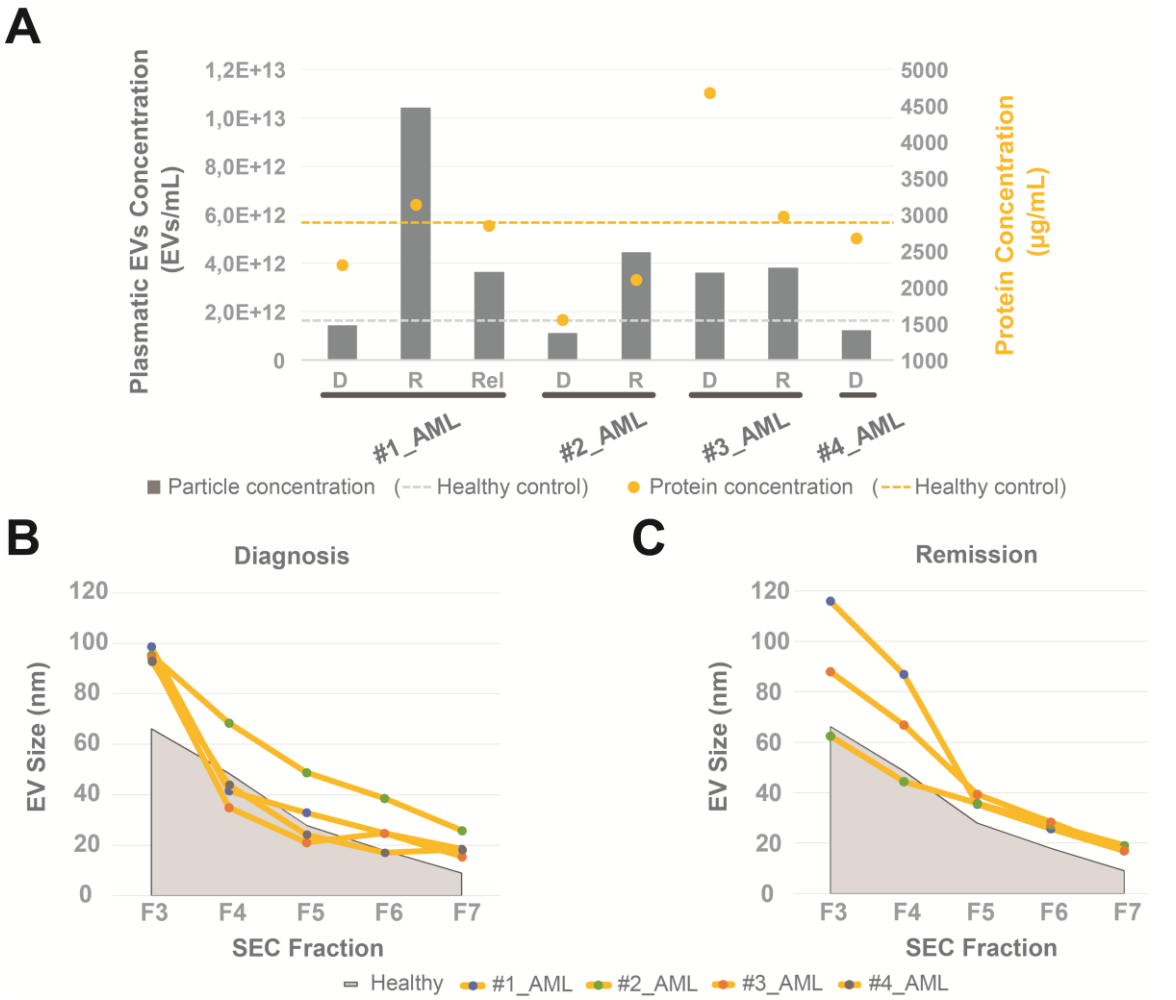

Figure 6 – AML patients presented higher concentrations of EVs at complete remission when compared to diagnosis. EVs concentration (grey bars; left axis) plotted against protein concentration (yellow line; right axis) measured by NTA and Lowry method respectively, for the pool of fractions 3 to 7 from each AML patient's condition (D: diagnosis; R: complete remission; and Rel: relapse) and from healthy control. (B) Comparison of the average EV size in SEC fraction 3 to 7 isolated from AML patients' blood plasma (at diagnosis and at complete remission) and from a healthy control. ID of four AML patients: #1_AML, #2_AML, #3_AML and #4_AML.

Regarding the most frequent size of circulating EVs, preliminary results from the NTA analysis indicate that the EV size (mode) was slightly higher for the AML patients when compared with the healthy control (**Table 2**). Moreover, DLS analysis also seems to corroborate this as most of the AML patients' EVs, particularly the ones isolated at SEC fraction F3, were slightly larger than the healthy EVs (**Figure 6B-C**). However, the sample and control number need to be increased in order to allow conclusions to be taken regarding this type of analysis.

5. Clinically validated AML markers were detected in patients' circulating EVs

Next, this project sought to understand whether clinically-associated AML proteins were present in the patients' circulating EVs. The AML-associated protein profile of circulating EVs was obtained by WB analysis of pooled SEC fractions 3 to 7. Strikingly, commonly associated markers of AML disease, CD33, CD34, CD117, CD123, MPO, NPM1 and FLT-3 were detected in the circulating EVs from AML patients (**Figure 7**). Moreover, some of these markers have a variable expression over the AML clinical evolution.

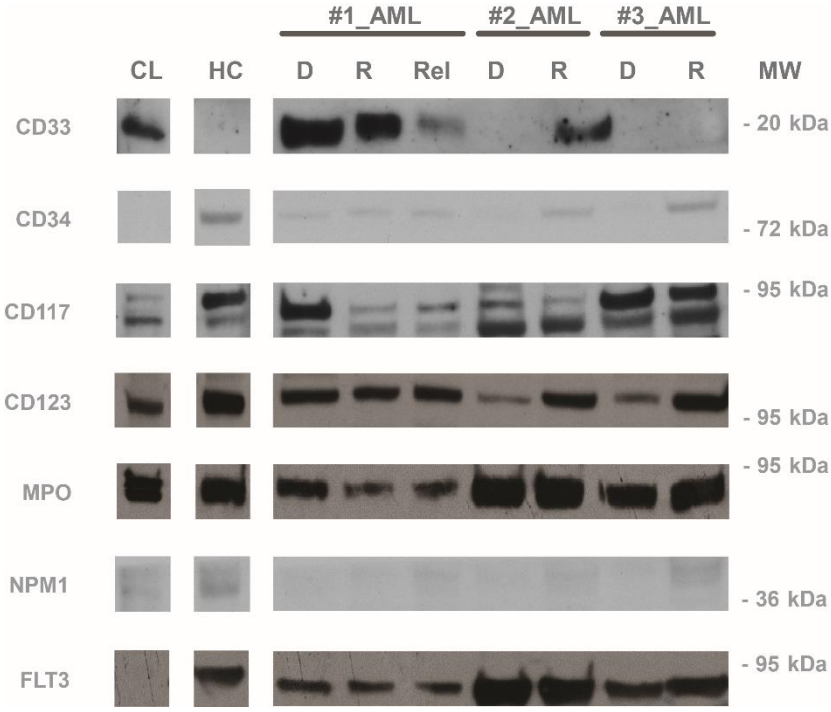

Figure 7 – AML protein markers were present in EVs isolated from AML patient's plasma. Analysis of proteins associated to AML (CD33, CD34, CD117 upper band, CD123, MPO, NPM1 and FLT3) in EVs isolated from samples (collected at diagnosis, complete remission and relapse) of AML patients and in a healthy control. Protein amounts obtained in healthy control fractions only made possible join a pool of fractions 6 and 7 in way to have sufficient protein amount to load in WB. CL: HL60 cell lysate; HC: healthy control; D: diagnosis; R: complete remission; Rel: relapse. Representative results obtained from three AML patients: #1_AML, #2_AML and #3_AML; and one healthy control. The gels loading was confirmed by Ponceau (Supplementary figure 3).

Results from the HC and from the CL allowed confirming the detection of the studied leukaemic markers by WB, using appropriate antibodies, having obtained bands with similar molecular weights to the ones published in the literature (Hong, Muller, Boyiadzis, et al., 2014; Tzoran et al., 2015). Most interestingly, all the studied leukaemic markers (usually detected in BM samples from biopsies) were detected in circulating plasmatic EVs. Moreover, in patient 1 the expression of most markers decreased following chemotherapy as expected. However, patients 2 and

3 presented an increase in some leukaemic markers (e.g. CD33, CD34, CD123) following chemotherapy. Two of the studied leukaemic markers, CD117 and MPO, presented decreased expression following chemotherapy but their expression was again increased at relapse.

6. Preliminary data: miR 150 was down-regulated in circulating EVs of AML patients

Additionally to the presence of clinically established AML protein markers, the miRs cargo of circulating EVs has been increasingly recognized as predictive biomarkers of leukaemic blast phenotype and therefore of AML prognosis (Caivano et al., 2016). Preliminary results show that could be detected miRs in circulating EVs from both patients and the control. Regarding the relative abundance of miRs that are often deregulated in AML (miR150 and miR155), preliminary data obtained by qRT-PCR indicated that the three AML patients presented less miR 150 packed into circulating EVs when compared with one healthy individual (**Figure 8**). Nevertheless, this observation was performed at high CT values (>30), due to the very low levels of miRs found in circulating EVs, precluding any meaningful interpretation of the data. In addition, the low number of patients and controls available did not allow taking further conclusions.

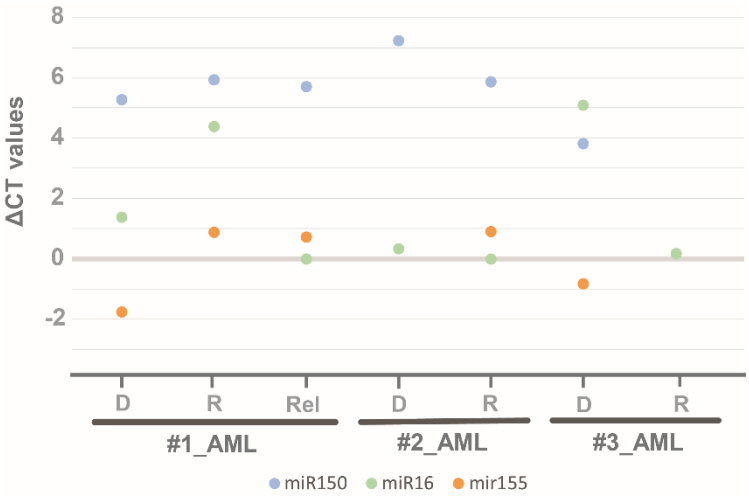

Figure 8 – miRs associated to AML were present in EVs isolated from AML patient's plasma. Analysis of miRs content associated with AML (miR150 and miR155) and miR16 as internal control present in EVs isolated from samples (collected at diagnosis, complete remission and relapse) of AML patients. The results are present as CT values variation in relation to healthy control. The CT values obtained by qRT-PCR were between 30 and 40. D: diagnosis; R: complete remission; Rel: relapse. Representative results obtained from three AML patients(#1_AML, #2_AML and #3_AML) and one healthy control.

General Discussion

MRD monitoring is a surveillance methodology that can improve clinical outcomes of patients. This is possible due to the ability of high-sensitivity methodologies to detect and measure specific disease markers in patients, making it possible to guide therapeutic interventions and anticipate relapse (Boyiadzis and Whiteside, 2016). In clinical practice, AML MRD detection is performed in BM samples. However, for sampling reasons, is not possible to do frequent monitorization. Therefore, the complementation of BM with PB samples analysis could allow a more frequent monitorization of patients. However, is necessary to optimize the methodologies to reach reliable sensitive levels.

As it was mentioned above, one of the possible approaches regarding MRD detection in PB samples, is the analysis of AML biomarkers presence in plasmatic EVs. These vesicles have the ability to carry cargo from the donor cells, making it possible to obtain information from BM cells by using a PB biopsy.

1. Intact and size-resolved EVs are isolated by Size Exclusion Chromatography from the peripheral blood of AML patients

The most widely used technique for EVs isolation is the ultracentrifugation, a method based in particles density. Nevertheless, this is a time-consuming technique that presents lack of reproducibility due to small variations in the protocol (Livshits et al., 2015). Moreover, the high centrifugal forces required to pellet the EVs most likely affects the vesicles' integrity. Indeed, this harsh process may promote EV disruption and/or their aggregation and fusion, compromising an unbiased EV profile analysis. Unfortunately, this process also pellets most of the protein "contaminants" present in the original sample, which is critical in the case of plasma-derived samples (Webber and Clayton, 2013; Linares et al., 2015).

Here, a SEC technology was implemented to isolate circulating EVs from the plasma of AML patients. This size-based separation technique is a cost-effective technology, does not require specialized infrastructures and has high reproducibility. Importantly, since SEC is a simple single-step EV isolation method, it is a fast way to isolate undamaged circulating EV from the plasma of patients, which is highly desirable in a hospital setting. Indeed, this work has shown that this method does not disrupt EVs and therefore minimally alters EVs characteristics as reported by others (Hong et al., 2016; Welton et al., 2015; Gamez-Valero et al., 2016; Mol et al., 2017). In this work, home-made SEC columns were used. For that,

the columns were prepared accordingly to *Anita Boing et al.* with some small alterations such as to remove the 0,45 µm filtration or the centrifugation at 10000 g.

This methodology was validated by identifying which fractions eluted the circulating EVs. From those analysis, fractions 3 to 7 were selected for downstream analysis due to the particle's size and concentration. Fractions 1 and 2 presented higher sizes (≥ 300 nm) although at a very low concentration when compared to the following fractions. To obtain PPP, the blood samples were centrifuged twice at 2500 g for 15 min at 18-20ºC. Although the majority of platelets and apoptotic bodies were removed during this initial plasma centrifugation, it was expected that reminiscent amounts from the PPP constituents' would most likely be eluted in first SEC fractions. Nevertheless, this hypothesis was not confirmed due to the very low particle concentration in these fractions. For these reasons, the first two fractions were not included in downstream analysis. Similarly, the later fractions (fractions 8 to 10) were also excluded from downstream analysis since they had very low size (≤ 8 nm in DLS) and very high amounts of total protein, suggesting that very high amounts of "contaminant" proteins were co-eluted with smaller EVs in these fractions. Altogether, these reasons prevented the proper analysis of the eluted EVs in fractions F1 to F2 and F8 to F10.Therefore, only fractions 3 to 7 were selected for unbiased EV downstream analysis. Despite the lower particle concentration, fraction 3 was included in downstream analysis. This fraction was included in further analysis since it presented a mean EV size of 128 nm, expression of actinin-4 mid-size EV marker together with the absence of cytochrome-c, consistent with the hypothesis that fraction 3 was enriched in microvesicle-like vesicles. On the contrary, fraction 7 was included in further analysis because the eluted particle size was still compatible with smaller EVs size. Noteworthy, reasonable amounts of free protein could be also co-eluted with these smaller EVs, as observed in TEM images and in the WB analysis of albumin expression.

The biological confirmation that EVs had been eluted from the SEC fractions was effectively provided by TEM and WB analysis. TEM photographs revealed that the particles eluted in fractions 3 to 7 had a morphology that resembled the lipid nature of EVs. Indeed, 3 types of morphology were found. Although most of these particles presented an intact round shape morphology, some presented a cup-shape while others displayed an irregular shape. The cup-shape was already shown to be a technical artefact due to the uranyl counter-staining. This hypertonic solution has

been described to cause cytoplasm output from the EVs, originating the "cup-shape", a wither morphology (Raposo and Stoorvogel, 2013). The small amount of particles found with irregular shape could be damaged EVs, most likely due to the conditions of the plasma storage and/or the TEM counter-staining methodology. However, it is not possible to exclude the possibility that these might be lipid bodies such as HDL or cholesterol(Boing et al., 2014).

The fact that the particles observed in fractions 3 to 7 were positive for the expression of CD63 and HSP70 further confirmed the presence of EVs. Indeed, those proteins are already well accepted in the literature as being EVs' markers (Colombo et al., 2014; Akers et al., 2013). Moreover, the absence of cytochrome C assured that these lipid-like particles were not cell debris membranes. Unfortunately, these EVs were co-eluted with minor amounts of albumin, a plasma "contaminant" protein, which is quite visible in fraction 7 both by TEM imaging and WB analysis. Thereby, the implemented home-made SEC technology was able to isolate biologically intact and size resolved circulating EVs from blood plasma of AML patients, though the later fractions co-elute high amounts of plasma proteins.

2. Recently reported size-dependent EV markers are differently expressed in the eluted SEC fractions

The EV sub-populations have different biogenesis and, for that reason, it is natural that they carry different cargo. Interestingly, the EV biogenesis seems to be altered in cancer cells, having an increase of EV shedding (Fais et al., 2016). Therefore, this work sought to understand whether specific EV sub-populations could be isolated in the different SEC fractions. This might be an important aspect for future studies since specific EV sub-populations (e.g. microvesicles or exosomes) may carry specific AML-related molecular signatures with prognosis interest. To decipher whether the SEC methodology was capable of eluting specific EV sub-populations in the different fractions, WB analysis was carried out with markers recently reported to be specific for small (annexin XI) or mid-size (actinin-4 and mitofilin) EVs (Kowal et al., 2016). Even though fractions 3 and 4 only presented mid-size markers (**Figure 5**), it seems that later fractions presented both types of size-makers. This might suggest that a mixture of these sub-populations is co-eluted in later fractions. Nevertheless, there might be other explanations for these results, as explained next. EVs were isolated accordingly to their size and it

was confirmed that larger EVs were eluted first while smaller EVs were co-eluted later together with some "contaminating" proteins (**Figure 4**). Arguably, the detection of mid-size markers in the late fractions could be due to the existence of these proteins (actinin-4 and mitofilin) co-eluted as free proteins and not associated to EVs. Another plausible reason is the fact that these size-markers were validated by a sucrose gradient ultracentrifugation (Kowal et al., 2016). Since the methodology used by those authors separates the EVs based on particle density (not size), their classification should be of high versus low density EVs. This might be important since EVs with the same size may have different biogenesis, and therefore the lipid constitution of these EVs membranes could be different. Indeed, this could originate EVs with the same size but with different densities, providing a more plausible explanation for the results here described for the SEC methodology.

Since in some fractions eluted from the SEC columns there is most likely a continuum of these EV sub-populations, downstream analysis was performed with pools of the fractions 3 to7.

3. Preliminary results suggest that AML patients have a higher concentration of plasmatic EVs at complete remission

In the last years, the potential use of circulating EVs as a source of cancer biomarkers called the attention of the scientific community. Interestingly, several of those studies reported that a higher concentration of circulating EVs were found in the blood plasma of cancer patients, when compared to healthy controls (Fais et al., 2016; Szczepanski et al., 2011; Logozzi et al., 2009).

Considering this, this work sought to understand whether there were differences on the plasmatic concentration and size profile of circulating EVs from AML patients, when compared to healthy controls and also over the course of AML treatment and clinical follow-up. Contrary to the literature, the preliminary data here presented did not show a higher EV concentration in the plasma of AML patients when compared to a healthy control, except for patient #3_AML. In general, the AML patients at diagnosis exhibited a lower plasmatic EV concentration when compared with the healthy control. However, the results here presented show that the plasmatic EV concentration is drastically increased at the patients' blood upon complete remission. This finding could be related with the induction

chemotherapy. Indeed, several studies point out that cellular stress, as the one induced by chemotherapy, increases the exosome release (Kanemoto et al., 2016; Desdin-Mico and Mittelbrunn, 2017). In fact, Xiao *et al*. reported that treatment of lung cancer cells with cisplatin originated an increase in exosome release by these cells (Xiao et al., 2014). Nevertheless, in the present thesis only 4 patients and 1 healthy control were analysed. Therefore, it is necessary to increase the number of patients and healthy controls analysed before drawing general conclusions.

Although DLS is a high sensitivity technique it is not a reliable for solutions with high polydispersity levels, such complex biological solutions. Nevertheless, DLS was used because SEC technology was able to resolve EVs by their size. Indeed, and although some overlapping EV size between EVs eluted in adjacent fractions were observed, the polydispersity index obtained was acceptable for a reliable analysis. As expected, when pooled fractions were analysed, the polydispersity index was too high for a trustworthy particle size analysis. In those cases, exceptionally, the EV size profile of the pool was evaluated by the size mode obtained by the NTA analysis. Although no general conclusion can be drawn due to the small patient cohort analysed here, it seems that patients with a poor AML clinical prognosis display a slightly higher size mode (80 nm) than the healthy control (63 nm). On the other hand, patients #3_AML and #4_AML which had a favourable AML prognosis had an EV size mode (65 nm) similar to the healthy control. It would be important to confirm these findings in a larger patient data-set.

4. Clinically validated AML markers are detected in patients' circulating EVs

MFC is one of the most widely used techniques for MRD assessment. Indeed, immune-phenotype analysis has a high impact on elucidating which lineage was affected by the leukaemic transformation and more importantly on MRD measurement. With that on mind, already knowing *a priori* which AML markers the patients presented in the MFC analysis, it was sought to understand whether some of these markers, that were phenotypically expressed by the patients' leukaemic blasts, were also expressed in the patients' circulating EVs.

This work shows that it is possible to detect the presence of all studied AML markers in the patients' circulating EVs. Importantly, even though absolute quantification is not possible in WB analysis, some of these markers presented a

variable expression throughout the course of treatment and clinical follow-up. An example is the protein CD117 (c-kit). Indeed, despite being present in the health control, the relative expression of CD117 seems to associate with disease development. In general, CD117 expression decreased at complete remission when compared to the corresponding paired diagnosis samples in all patients. Moreover, the referred marker presented an increase in expression in the relapse sample of patient #1_AML.

In addition, in patient #1_AML most of the analysed AML markers decreased their expression after the induction chemotherapy (complete remission samples). This could reflect an eventual decrease of leukaemic cells, causing a decrease in the sheding of leukaemia-derived EVs positive for these AML markers in circulation. Interestingly, comparison of the intensities obtained at complete remission (Figure 7) with particle concentration data (Figure 6) for this patient #1_AML, further strengthens this hypothesis since the increase in the number of EVs in circulation at complete remission was not associated with an increase in the presence of leukaemic markers.

However, patients #2_AML and #3_AML presented the opposite results, given that some AML markers were increased in complete remission. Since the clinical data from those patients show a decrease in the leukaemic burden, and the patients remain in remission, the increase in the levels of these markers in the circulating EVs is probably associated with other factors not related to leukaemia. Therefore, to obtain a justification for this tendency, it is necessary to do more protein analyses.

Taken together, the analysed AML markers, especially CD117 and MPO, could be of potential interest to predict relapse in EV-based MRD monitoring. However, in spite of the here shown potential of EV-based liquid biopsy for MRD assessment, further studies with larger patient numbers are needed to attest the real clinical value of this approach.

5. Preliminary data: miR 150 is down-regulated in circulating EVs of AML patients

Another field with an increasing interest by the scientific community is the cancer epigenetic landscape and the miRs study. It is accepted that EVs carry

nucleic acids, including miRs (Colombo et al., 2014). Moreover, it has been reported that specific miRs such as miR150 and miR155 are deregulated in AML (Caivano et al., 2016). Here, miRs were isolated from the isolated EVs. Although preliminary results suggest that there might be a downregulation of miR150 in AML samples when compared with the healthy control, the amount of extracted miRs from the isolated EVs was too low to allow obtaining reliable results. Therefore, these results need further confirmation.

6. Experimental pitfalls and other considerations

Although this thesis work was performed with clinically valuable AML human blood samples, some potential drawbacks should be acknowledged. The plasma isolation with a centrifugation speed of 2500 g is described to obtain PPP (Saenz-Cuesta et al., 2015). However, it should be noted that this sample preparation step could impact the obtained results. For instance, some larger EV subpopulations could be lost from downstream analysis due to the referred sample preparation for SEC. Therefore, the use of lowers centrifugation speed could potentially increase the quantity of large to mid-size EVs isolated in fractions 1 to 3.

In TEM, the irregular shape bodies identified as EVs could also be large lipid bodies such HDL and cholesterol. This matter would require a further examination to confirm this hypothesis, by performing lipid quantification in the fractions to know if there are high variations between these earlier fractions and later fraction where these lipid particles were not observed. This approach would need to be normalized by particle number (provided by NTA analysis). Moreover, it would be interesting to also demonstrate that the observed particles in TEM are positive for specific EVs or even AML markers, by immuno-electron microscopy.

The WB analysis was a challenging task for EV characterization in the isolated plasma-derived EVs. Indeed, the fact that the isolated fractions had the EVs diluted in 1 mL was a problem. With that dilution, it was not possible to obtain enough protein to perform WB. To circumvent this problem, the SEC fractions were concentrated by speed vacuum technology, commonly used in proteomic studies, prior to the WB analysis. In addition, the usual loading controls (actin or tubulin) did not correlate with the total protein loaded (since the protein cargo of EVs might not include these proteins in equal amounts). Thus, Ponceau staining was used as the best available loading control. Therefore, the WB only provided a broad analysis

of protein amounts in different EVs. To establish AML threshold levels for specific proteins in EVs more sensitive technologies would be required, such as MFC or even high-throughput proteomic technologies.

For analysis of miRs by qRT-PCR, an optimization of the protocol was performed. Nevertheless, similarly to the WB, the dilution of the eluted EVs became a major limitation. The quantity of miRs extracted was too low, originating the amplification of the selected miRs at very high CTs values. Therefore, future miR quantification studies should either concentrate the sample (without causing miRs degradation) or rely on alternative plasma isolation protocols. Indeed, the protocol used in this thesis for the isolation of blood plasma started with a dilution of 1 mL of blood plasma in 20 mL of 0.32% Sodium Citrate in PBS. This protocol should be reviewed in order to allow less dilution of the EVs in the eluted fractions.

7. Future perspectives

Here is demonstrated that AML patients' circulating EVs carry clinically established AML markers. Even though precise protein expression analysis was not possible (due to technical problems associated with EVs), this work demonstrated that some proteins are differently present in patients' circulating EVs throughout the course of leukaemia treatment and clinical follow up. So, it would be interesting to perform deep quantitative high throughput proteomic analysis in isolated EVs, focused on AML markers and possibly other markers.

Another important aspect that would be interesting to pursue is the isolation of a specific sub-population of leukaemia-derived EVs. This might be possible by affinity chromatography, with antibodies for some of the AML markers found to be present in the circulating EVs. This would allow performing a more specific characterization of these leukaemia blast-derived EVs and comparison to healthy controls. Moreover, affinity chromatography allows sample elution in lower volumes than SEC. This would be a major advantage for an easier downstream analysis.

Conclusion

The identification of a differential EV profile for AML patients who are at higher risk for relapse will grant the EV-based MRD detection technology great promise to impact cancer in all stages of development and clinical practice.

Here, it was possible to isolate plasma-derived EVs from AML patients' peripheral blood by SEC. This technique was successfully implemented in the laboratory and properly validated for the isolation of intact circulating EVs. The validation of the SEC protocol for intact EVs isolation was possible by using a set of methodologies such as DLS, NTA, TEM and WB. Most of the EVs were found in fractions 3 to 7, where fractions 3 and 4 presented only mid-size EVs, whereas the following fractions presented a mix of mid and small-size EVs. With this EV isolation methodology it was possible to isolate the circulating EVs without evident co-isolation of cell debris. Nevertheless, albumin was still present in the eluted fractions, showing the presence of "contaminant" proteins.

Importantly, it could detect some of the clinically validated AML markers in the AML patients' circulating EVs and characterize their relative abundance in the blood of AML patients throughout the clinical follow up. Indeed, the quantity of some of these AML markers, such as CD117 (c-kit), present in the EVs, seems to vary with clinical development, making them potential biomarkers to predict relapse. Nevertheless, is needed the confirmation of this in a larger patient data-set before drawing definitive conclusions.

In summary, this work illustrates the utility of SEC to isolate intact EVs, and how it could be implemented in the clinical practice. In addition, this work proved that it is possible to detect AML markers in circulating EVs from AML patients. Moreover, some of these markers, such as CD117, could be potential AML MRD biomarkers on EV based liquid biopsies.

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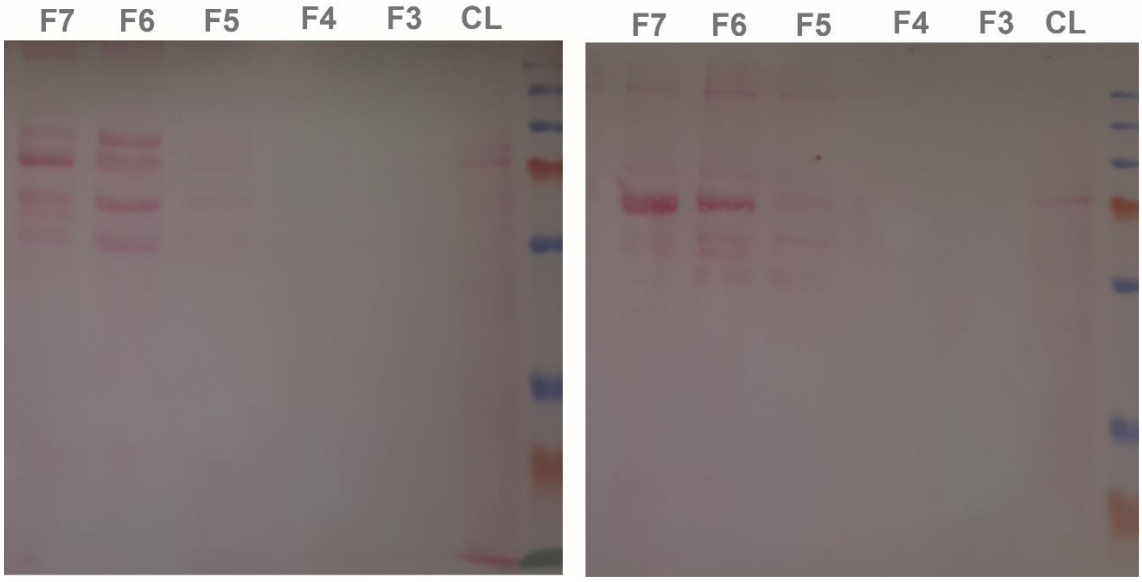
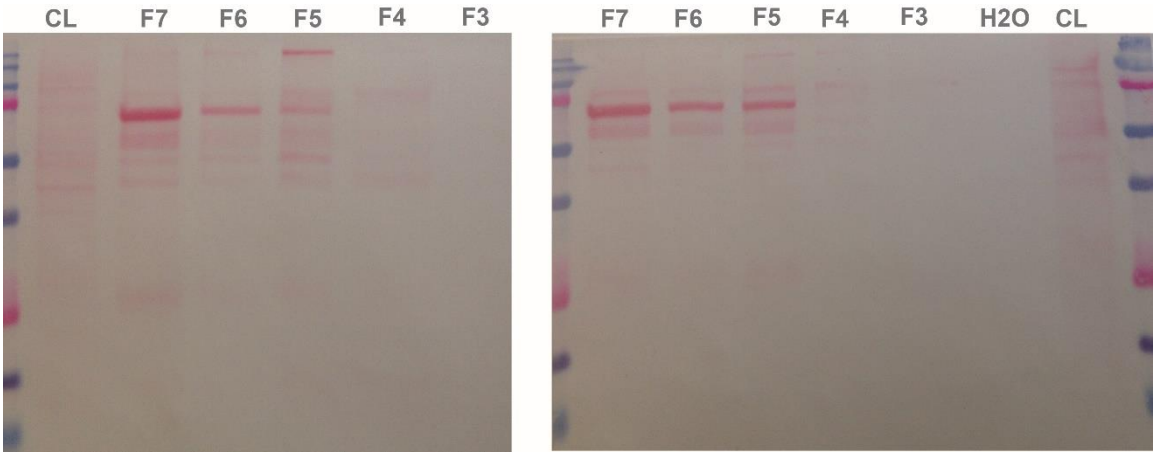
Supplementary Material

Supplementary figure 1



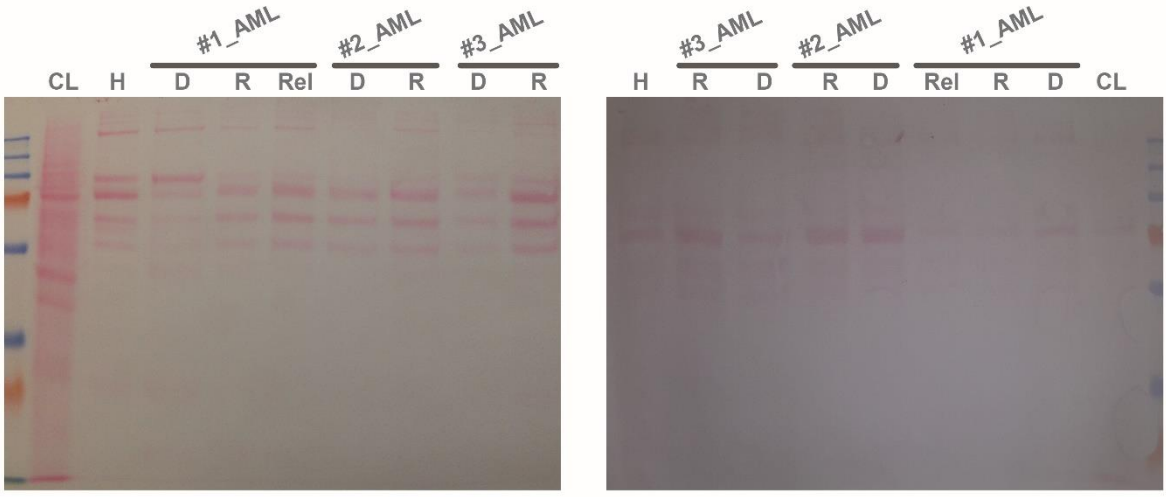
Supplementary figure 1 – Ponceau protein staining for confirmation of loading. Ponceau staining of the EVs fractions 3 to 7 in analysis of EV markers (CD63 and HSP70) and in EV purity (cytochrome c and albumin). It shows that different amounts of protein were loaded in each lane. CL: HL60 cell lysate

Supplementary figure 2



Supplementary figure 2- Ponceau protein staining for confirmation of loading. Ponceau staining of the EVs fractions 3 to 7 in analysis of different EVs sub-populations (Actinin-4, mitofilin and annexin XI). It shows that different amounts of protein were loaded in each lane. CL: HL60 cell lysate; H2O: lane filled only with water instead of protein sample.

Supplementary figure 3



Supplementary figure 3- Ponceau protein staining for confirmation of loading. Ponceau staining of the pool EVs isolated from samples (collected at diagnosis, complete remission and relapse) of AML patients and in a healthy control. These membranes were used to analyses AML markers (CD33, CD34, CD117, CD123, MPO, NMP1 and FLT3). CL: HL60 cell lysate.